Bioactive products formed in humans from fish oils.

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Abstract

Resolvins, maresins and protectins can be formed from fish oils. These specialized pro-resolving mediators (SPMs) have been implicated in the resolution of inflammation. Synthetic versions of such SPMs exert anti-inflammatory effects in vitro and when administered to animal models. However, their importance as endogenous products formed in sufficient amounts to exert anti-inflammatory actions in vivo remains speculative.

We biased our ability to detect SPMs formed in healthy volunteers by supplementing fish oil in doses shown previously to influence blood pressure and platelet aggregation under placebo-controlled conditions. Additionally, we sought to determine the relative formation of SPMs during an acute inflammatory response and its resolution, evoked in healthy volunteers by bacterial lipopolysaccharide. Bioactive lipids, enzymatic epoxyeicosatrienoic acids and free radical-catalyzed prostanoids (isoprostanes) formed from arachidonic acid and the fish oils, served as comparators. Despite the clear shift from ω-6 to ω-3 epoxyeicosatrienoic acids and isoprostanes, we failed to detect a consistent signal in most cases of SPM formation in urine or plasma in response to fish oil and in all cases in response to lipopolysaccharide on a background of fish oil.

Our results question the relevance of these SPMs to the putative anti-inflammatory effects of fish oils in humans.

Introduction

The clinical cardiovascular utility of a diet rich in fish oils, particularly eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), has been debated over the past 50 years (1-3). Large clinical outcome trials, such as the open-labeled GISSI-Prevenzione (4) or JELIS (5) studies, supported the notion that fish oil supplements confer therapeutic benefit on patients with cardiovascular disease. However, an overview analysis of results of more than 50 randomized controlled trials and cohort studies addressing this question yielded equivocal results (6, 7). Consequently, the adoption of the dietary interventions with fish oil into clinical guidelines has been limited (8). Fish oil supplementation does influence a series of cardiovascular biomarkers – it decreases blood levels of triglycerides in patients with hypertriglyceridemia, an effect primarily driven by lowering the production of triglycerides from non-esterified fatty acids (9); high doses reduce blood pressure in patients with essential hypertension (10, 11), and inhibit modestly indices of platelet activation (12). The mechanisms involved are unclear, but may involve a shift in formation of enzymatic and free radical catalyzed prostanoids, reflecting utilization of EPA and DHA rather than arachidonic acid (AA) as a substrate. It has been speculated also that cardiovascular benefit might derive in part from anti-inflammatory actions of fish oils: families of bioactive lipids which favor resolution of inflammation have been suggested to be of particular importance (13). Synthetic versions of such specialized pro-resolving mediators (SPMs), products of transcellular metabolism of fish oils, exert anti-inflammatory effects in vitro and when administered in vivo in several animal models (14-17). Quantities of exogenous SPMs in these models, however, are substantial, 0.6 μg Resolvin (Rv) D1 per mouse for example was used to attenuate the lipopolysaccharide (LPS) -induced inflammatory response in lung (18). These quantities are in marked contrast to the low pg/ml concentrations reported in humans (19).

Thus, the importance of SPMs as endogenous products formed in sufficient amounts to exert an anti-inflammatory action in vivo remains speculative. A particular limitation has been the use of assays in biological systems related to capacity – such as serum - rather than actual biosynthesis in a field where a marked discrepancy between the two approaches has long been recognized (20). Here, the fact that detection of SPMs is confounded by cells activated *ex vivo* in the test tube often remains unrecognized. An additional limitation has been of a technical nature. There are few data attesting to their formation in humans based on rigorous mass spectrometric methodology. Most clinical investigations reporting the formation of SPMs rely on immunoassays (21) or mass spectrometry without synthetic internal standards structurally similar to the SPMs under investigation (22-32) often in *ex vivo* stimulated systems (17, 33-36).

Here we biased our ability to detect SPMs formed in healthy volunteers by administering to them high doses of fish oils, such as have been shown to influence blood pressure and platelet aggregation under placebo controlled conditions (11, 12). As a comparator, we also analyzed enzymatic (epoxyeicosatrienoic acids [EETs]) and free radical (isoprostanes [iPs]) catalyzed prostanoids formed from AA and the fish oils. Additionally, we sought to determine the relative formation of SPMs and these bioactive lipids during an acute inflammatory response and its resolution, evoked in healthy volunteers by administration of LPS. Despite a clear shift from ω-6 to ω-3 EETs and iPs, we failed to detect a consistent signal in most cases of SPM formation in urine or plasma in response to fish oil supplementation and in all cases in response to LPS on a background of fish oil supplementation.

Methods

Human Studies

Samples were available from a human study (clinicaltrials.gov registration number: NCT00682318) assessing the interaction of high doses of marine lipids delivered as Lovaza fish oil with ethanol on lipid peroxidation. Healthy volunteers (n=12) received, in an open single-arm design, 7 capsules Lovaza^{TM} TID for 24.2±2.3 days (Figure 1a). This dose delivered a total of 17.6 g/day ω-3 PUFA, consisting of 55.1% EPA (9.7 g/day) and 44.9% DHA (7.9 g/day). Prior to study enrollment, informed consent, approval by the Institutional Review Board and authorization by the FDA (IND#79,750) had been obtained. Subjects were non-smokers, not pregnant and abstained from the use of high-dose vitamins, NSAIDs, and illicit drugs as assessed by cotinine (Craig Medical, Vista, CA) and pregnancy tests, history, platelet aggregometry (37), and a urine drug screen (RDI, Poteau, OK), respectively, for at least two weeks before enrollment and throughout the study. Health status and safety was assessed by routine medical history, physical exam, and laboratory work (hematology, biochemistry, and urinalysis) at time of screening and on completion of the study. Subjects were counseled not to make major changes in their diet, and to refrain from consuming any additional fish foods to minimize exposure to contaminant heavy metals including mercury, apparent in fish products. Compliance with this regimen was assessed daily by real-time text messaging or emailing the intake of Lovaza^{TM} capsules, weekly by capsule count in the Clinical and Translational Research Center (CTRC), by the change of lipid ratios in red blood cell membranes (Figure 1c) and diversion from AA-derived isoprostanes and epoxides towards EPA-/DHA-derived species (Figures 1d-h).

Plasma samples were also available from a subset of healthy volunteers (n=6), studied to evaluate the systemic inflammatory response to experimental endotoxemia in subjects treated

with lower doses of marine lipids delivered as Lovaza fish oil; please refer to reference (38) for further details (clinicaltrials.gov registration number: NCT01048502). In brief, this group of subjects was randomized to receive 2 capsules Lovaza^{TM} BID for 8 weeks in a double-blinded fashion, followed by an intravenous bolus of U.S. standard reference endotoxin (LPS; lot No. CCRE-LOT-1 +2; Clinical Center, Pharmacy Department at the National Institutes of Health, Bethesda, MD, USA) dosed at 0.6 ng/kg body weight (Figure 2a).

A pilot study in 21 healthy participants, established baseline values of urinary 5-*epi*-8,12-*iso*-iPF$_{3\alpha}$-VI; 8,12-*iso*-iPF$_{2\alpha}$-VI; and iPF$_{2\alpha}$-III. All studies were approved by the Institutional Review Board and informed consent was obtained from the participants prior to study initation.

Biospecimens

Blood (Plasma): For the study *"High doses of Lovaza fish oils"* venous blood was drawn into Na-heparin vacutainer tubes after an overnight fast on six occasions: two times as baseline (12.2±6.0 and 3.0±0.0 days before start of fish oil supplementation), two times during fish oil supplementation (12.0±0.0 and 24.2±2.3 days after the start of fish oil supplementation), and two times after discontinuation of fish oil supplementation (7.9±0.3 and 13.3±0.8 days after stop of taking fish oil). Plasma was separated by immediate centrifugation (3000 RPM, 15 minutes, 4°C) and stored at -80°C until extraction.

For the study *"Evoked Endotoxemia"* venous blood was drawn into citrate vacutainer tubes after an overnight fast on seven occasions: one time as baseline (before the start of fish oil supplementation), one time after 50.0±6.8 days of fish oil supplementation (which coincided as baseline prior to the injection of LPS), and five times after the intravenous injection of LPS (time course of 2, 6, 12, and 24 post LPS administration as well as a follow-up any time between 48-72

hours post-LPS). Plasma was separated by immediate centrifugation (3000 RPM, 15 minutes, 4°C) and stored at -80°C until extraction.

Blood (Red Blood Cell Membranes): For the study *"High doses of Lovaza fish oils"* venous blood was drawn into EDTA vacutainer tubes after an overnight fast on six occasions: two times as baseline (12.2±6.0 and 3.0±0.0 days before start of fish oil supplementation), two times during fish oil supplementation (12.0±0.0 and 24.2±2.3 days after the start of fish oil supplementation), and two times after discontinuation of fish oil supplementation (7.9±0.3 and 13.3±0.8 days after stop of taking fish oil). The tubes were immediately centrifuged at 3000 RPM for 15 minutes (4°C) to separate the red blood cells (RBCs) from plasma and the buffy coat. RBCs were transferred to a clean tube and washed twice with ice-cold isotonic saline, followed each time by centrifugation for 10 minutes (3000 RPM, 4°C). Washed red blood cells were stored in saline (33:67 v/v) at -80°C until extraction. For the study *"Evoked Endotoxemia"* red blood cells were obtained from venous blood as described in (38).

Urine: For the study *"High doses of Lovaza fish oils"* 12-hour overnight urine samples were collected on eight occasions: two times as baseline (12.2±6.0 and 3.0±0.0 days before start of fish oil supplementation), four times during fish oil supplementation (5.0±0.0, 12.0±0.0, 19.1±0.3 and 24.2±2.3 days after the start of fish oil supplementation), and two times after discontinuation of fish oil supplementation (7.9±0.3 and 13.3±0.8 days after stop of taking fish oil). Urine aliquots with quality control samples were stored until extraction at -80°C.

Mass Spectrometry

Materials. d_4-RvE1, d_2-RvD1, d_2-PD1, and d_2-Maresin were synthesized and kindly furnished by Dr. Bernd Spur (39, 40). Maresin, RvD1, RvD2, PD1, 5(6)-EET, d11-5(6)-EET, 8(9)-EET, d_{11}-8(9)-EET, 11(12)-EET, d_{11}-11(12)-EET, 14(15)-EET, d_{11}-14(15)-EET, 8(9)-EpETE, 11(12)-EpETE, 14(15)-EpETE, 17(18)-EpETE, 16(17)-EpDPE, 19(20)-EpDPE, AA, d_8-AA, EPA, d_5-EPA, DHA, and d_5-DHA were purchased from Cayman Chemical Company (Ann Arbor, MI). We verified that the deuterated internal standards for EETs, DHA and EPA are not contaminated by authentic unlabeled lipids (Supplementary Figure 6a). Please note that Protectin D1 (PD1) and Neuroprotectin D1 (NPD1) are structurally equivalent, while Protectin DX (PDX) is an isomer (41). d_3-creatinine was purchased from CDN Isotopes (Quebec, Canada). Synthetic, d_4-8,12-*iso*-iP$F_{2\alpha}$-VI, and d_4-5-*epi*-8,12-*iso*-iP$F_{3\alpha}$-VI were synthesized by Joshua Rokach, PhD, Florida Institute of Technology, Melbourne, FL, as previously described and used as internal standards (42-46). Burdick and Jackson solvents were purchased from Honeywell. All water was freshly-generated Millipore, 18.2 MΩ.

Sample preparation. Plasma: To one ml of plasma was added a mix of deuterium-labeled internal standards in 50 μl acetonitrile (AcN). Included in the internal standard was 1 ng each d_4-RvE_1, d_{11}-5(6)-EET, d_{11}-8(9)-EET, d_{11}-11(12)-EET, d_{11}-14(15)-EET and 0.2 ng each d_2-RvD1, d_2-PD1, d_2-Maresin. The sample was gently mixed and allowed to equilibrate for 15 min. Formic acid, 50 μl, was added immediately before applying the sample to a solid phase extraction cartridge (StrataX, 30 mg; Phenomenex, Torrance, CA) that had been conditioned with 1 ml AcN followed by 0.25 ml water. The cartridge was washed with 1 ml water, dried by application of vacuum, and eluted with 1 ml 50% AcN/ethyl acetate (EtOAc). The eluate was dried under a

stream of N_2 and dissolved in 15 µl AcN. For analysis, 135 µl water was added and 75 µl was injected into the UPLC/MS/MS. **Urine:** Deuterium-labeled internal standards dissolved in 50 µl AcN were added to 2 ml of urine, gently mixed, and allowed to equilibrate for 15 min. The internal standard consisted of 5 ng each d_4-RvE1, d_2-RvD1, d_2-PD1, and d_2-Maresin. The sample was then acidified with 20 µl formic acid and applied to a StrataX SPE cartridge which was washed and eluted as described above. Isoprostanes were quantitated as described previously (47). In brief, 5 ng of each internal standard was added to 1 ml of urine. The solid phase extraction cartridge was conditioned with 1 ml of acetonitrile (AcN) and equilibrated with 0.25 ml of water. The sample was applied to the cartridge, which was then washed with 1 ml of 5% AcN in water and dried with vacuum for 15 min. The analyte and internal standards were eluted from the cartridge using 1 ml of 5% AcN in ethyl acetate. The eluate was collected and dried under a gentle stream of nitrogen. The resulting residue was reconstituted in 200 µl of 20% AcN in water and filtered by centrifugation using 0.2-µm Nylon Microspin filters (Spin-X HPLC Filter Fisher Scientific). 100 µl was injected. Metabolite levels were corrected for urinary creatinine. **Urinary Creatinine:** To 20 µl urine was added 1 ml AcN containing 10 µg d_3-creatinine. Ten µl of this solution was injected into the LC/MS/MS. Mobile phase A consisted of AcN, mobile phase B of 5 mM ammonium format at pH 4.0. A Waters, XBridge BEH HILIC 2.1 x 50 mm x 2.5 um was used as the LC column. The LC was programmed at 3.5% mobile phase B at 350 ul/min flowrate. **Lipids from red blood cell membranes:** Lipids were extracted using a modified Folch method (48). 50 µL of red blood cells (RBC), mixed in 1:3 ratio with saline, were used per sample, and further diluted with an additional 950 µL of saline after thawing from freezer. The RBCs were added to an internal standard (dried using nitrogen gas) containing labeled fatty acids (d_8-arachidonic acid, d_5-EPA, and d_5-DHA). The red cells were homogenized

in chloroform and methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant. The samples were then base hydrolyzed (KOH 7.5% in H_20) for 30 minutes. The pH was adjusted with formic acid to ~2.5. The samples were purified by solid phase extraction (SPE).

LC/MS/MS Analysis. All lipid analyses were performed on a Xevo TQ-S tandem mass spectrometer interfaced with an Acquity Ultra Performance Liquid Chromatograph (UPLC; Waters, Milford, MA), except for the analysis of urinary isoprostanes which were run on a TSQ Quantum Ultra instrument (Thermo, Waltham, MA; please see instrument parameters below)). The mass spectrometer was operated in the negative ion electrospray ionization (ESI) multiple reaction monitoring (MRM) mode using argon as the collision gas. The ESI probe capillary potential was 2.8 kV. The source was maintained at 150^o C, the desolvation temperature was 350^o C. All samples were chromatographed on a BEH C18 UPLC column (150 mm x 2.1 mm x 1.7 μ particle size; Waters) with a flow rate of 350 μl/min. Mobile phase A consisted of Millipore water containing 0.05% acetic acid, adjusted to pH 5.7 with ammonium hydroxide. Mobile phase B was AcN/MeOH (95/5). Solvent AcN Optima ® is from Fisher Scientific, while MeOH is from Burdick & Jackson, HPLC grade, meets ACS specifications.

LC/MS/MS Analysis of SPMs in Urine. The UPLC gradient was held at 10% B for one min., followed by a linear gradient to 22% at 10 min., 38% at 13 min, and 48% at 20 min. The column was then washed for 2 min. with 100% B before returning to initial conditions which were held for a 5 min. equilibration period. Transitions monitored were m/z 349→m/z 161 (RvE1), m/z 353-m/z 162 (d_4-RvE1) collision energy (CE) 18 eV; m/z 375→m/z 141 (RvD1/RvD2), m/z

377→m/z 141 (d_2-RvD1) CE 14 eV; m/z 359→m/z 153 (PD1), m/z 361→m/z 153 (d_2-PD1) CE 15 eV; m/z 359→m/z 177 (Maresin), m/z 361→m/z 179 (d_2-Maresin) CE 14 eV.

LC/MS/MS Analysis of Isoprostanes in Urine. The HPLC included an Accela solvent delivery system (Thermo, Waltham, MA) and a Hypersil GOLD C18 (2), 200 mm × 2.1 mm, 1.9 μm particle size column (Thermo). The mobile phase consisted of water (solvent A) and acetonitrile: methanol (95:5, solvent B), both with 0.005% acetic acid adjusted to pH 5.7 with ammonium hydroxide. The flow rate was 350 μl/min. The mobile phase gradient began at 5% B, increased linearly to 18.5% B at 45 min, then to 38% B at 65 min. A TSQ Quantum Ultra instrument (Thermo) equipped with a heated electrospray source, and a triple quadrupole analyzer was used in these studies. The ESI source used nitrogen for both sheath and auxiliary gas, set to 70 and 0 arbitrary units, respectively. The mass spectrometer was operated in the negative ion mode with a capillary temperature of 350 °C and a spray voltage of 0 kV. The source offset was 6 V. The analyzer was operated in the multiple reaction monitoring (MRM) mode for the analysis of urinary iPs. The transitions monitored were: m/z 353 > 115 for the endogenous 8,12-*iso*-iP$F_{2\alpha}$-VI and m/z 357 > 115 for the corresponding tetradeuterated internal standard. The collision energy was 24 eV. For the endogenous 5-*epi*-8,12-*iso*-iP$F_{3\alpha}$-VI the transitions monitored were m/z 351 > 115 and m/z 355 > 115 for the corresponding tetradeuterated internal standard. The collision energy was 23 eV.

LC/MS/MS Analysis of SPMs/epoxides in Plasma. The UPLC was held at 10% B for one min., then programmed linearly to 22% at 10 min., 40% at 14.5 min, and 53% at 27.5 min. The column was then washed with 100% B for 2 min. and allowed to equilibrate at initial conditions

for 5 min prior to the next injection. Transitions monitored were those of urine (above) and m/z 319→m/z 191 (5(6)-EET), m/z 330→m/z 202 (d_{11}-5(6)-EET) CE 11 eV; m/z 319→m/z 155 (8(9)-EET), m/z 330→m/z 155 (d_{11}-8(9)-EET) CE 14 eV; m/z 319→m/z 167 (11(12)-EET), m/z 330→m/z 167 (d_{11}-11(12)-EET) CE 14 eV; m/z 319→m/z 257 (14(15)-EET), m/z 330→m/z 268 (d_{11}-14(15)-EET) CE 14 eV; m/z 343→m/z 233 (16(17)-EpDPE) CE 12 eV; m/z 343→m/z 299 (19(20)-EpDPE) CE 12 eV.

LC/MS/MS Analysis of Creatinine in Urine. A Thermo Ultima LC/MS/MS interfaced to an Accela UPLC (Thermo Scientific, Waltham, MA) was used in the positive ion chemical ionization (CI) MRM mode using argon as the collision gas. The MS interface capillary was held at 350^{o} C. The CI probe was set at 350^{o} C. The discharge current was 4.0 μA. The column was an XBridge BEH HILIC, 50 mm x 2.1 mm x 2.5 μ particle size (Waters). Mobile phase A was AcN; B was 100 mM ammonium formate, adjusted to pH 4.0 with acetic acid. The UPLC program was isocratic at 7.5% B. Transitions monitored were 114→86 (creatinine) and 117→89 (d_3-creatinine) at CE 12 eV.

LC/MS/MS Analysis of Lipids from Red Blood Cell Membranes. The analyzer was operated in the MRM mode for the analysis of the fatty acids EPA, DHA, and AA. The transitions monitored were m/z 301.2 > 257.2 for the endogenous EPA and m/z 306.2 > 262.2 for d_5-EPA; m/z 327.2 > 283.2 for the endogenous DHA and m/z 332.2 > 288.2 for d_5-DHA; m/z 303.2 > 259.2 for the endogenous AA and m/z 311.2 > 267.2 for d_8-AA. Collision energy was 12 eV for all compounds.

Limit of Detection Calculations

Neither RvD1, RvE1, nor Maresin were observed by UPLC/MS/MS in the study involving administration of high doses of Lovaza fish oils. To determine the actual limit of detection (LOD) of the samples in question, we identified and integrated one or more peaks with a signal-to-noise ratio of three or greater that eluted near the expected retention time for the compound in question. When the areas of these peaks are divided by the areas of the deuterated standards, we obtain a value in pg (Supplemental Figure 3a). Since no peak was observed at the correct retention time, the amount is therefore less than that of the selected peaks. For the RvD1 samples shown in Figure 1b the integrated peaks ranged from 3.4 pg to 4.3 pg. When the volume of the sample is taken into account, this calculates to a range of 3.9 to 4.2 pg/ml of plasma. For the RvE1 samples in Figure 1b, the limit of detection ranges from 7.8 to 10.4 pg/ml of plasma. For Maresin, the limit of detection ranges from 3.4 to 3.6 pg/ml of plasma. A limit of detection of 0.2-0.3 pg/mL of plasma was approximated for PD1 using the signals depicted in Figure 1. Noise was calculated as root mean square (RMS) from selected time windows in the chromatograms. The limit of detection was then extrapolated to discriminate a signal from noise at a ratio of 3:1.

In the study of lower dose Lovaza fish oils in which volunteers were administered LPS, RvD1 and RvD2 were not observed. Calculations analogous to those above show the limit of detection for the RvD1 samples shown in Figure 2b to range from 3.4 to 6.1 pg/ml of plasma. For the RvE1 the range is from 6.8 to 10.0 pg/ml of plasma. The limit of detection for PD1, based on the signals of Figure 2 and determined as described above, ranged from 0.1-0.3 pg/mL. Notably for one sample, the limit of detection was one order of magnitude lower at 0.02 pg/mL.

Recovery

The analytes with homologous internal standards were RvE1, PD1, Maresin, 5(6)-EET, 8(9)-EET, 11(12)-EET, and 14(15)-EET. The remaining non-polar compounds were quantitated against d_{11}-14(15)-EET, that is 8(9)-EpETE, 11(12)-EpETE, 14(15)-EpETE, 17(18)-EpETE, 16(17)-EpDPE, and 19(20)-EpDPE.

The recovery of each compound for which a homologous internal standard (IS) was available was calculated from the average area of the UPLC/MS/MS IS peak from each sample divided by the average area of two ISs that were not subjected to SPE. In detail, the recoveries amounted to 76% for RvE1; 77% for PD1; 71% for Maresin; 69% for 5(6)-EET; 81% for 8(9)-EET; 84% for 11(12)-EET; and 83% for 14(15)-EET. The recovery was not calculable for the compounds for which a homologous internal standard was unavailable.

Standard Curve

The relative UPLC/MS/MS response of compounds, for which d_{11}-14(15)-EET was used as a heterologous IS, was obtained by generating a six-point standard curve, 0, 1, 5, 10, 50, and 100 ng d_{11}-14(15)-EET in triplicate, in which the amount of d_{11}-14(15)-EET was constant and the amount of the target analyte varied (Supplemental Figure 5). The equations derived from the linear regression from these standard curves were used to adjust the response of the analytes in the actual samples.

Markers of inflammatory response

Details on the analysis of the panel of inflammatory responses to the administration of LPS, consisting of tumor-necrosis-factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10),

interleukin-1 receptor antagonist (IL-1RA), monocyte chemoattractant protein-1 (MCP-1), C-reactive protein (CRP), and serum amyloid A (SAA) have been published in (38).

Statistical Analyses

Descriptive statistics were executed in JMP® Pro Version 10. Means ± standard deviations are depicted unless otherwise stated. Averaged baseline measurements served as reference point to calculate percent changes from baseline. Tests of significance were not conducted due to the exploratory nature of this study.

Results

Human Subjects and Safety

Twelve subjects concluded the study *"High dose fish oils in volunteers"* (Supplemental Table 1); one subject discontinued due to personal reasons (CONSORT diagram in Supplemental Figure 1a). Three subjects deviated from the supplementation regimen of 7 capsules Lovaza™ TID; that is, subject ID #31 took 6 capsules instead of 7 on four occasions; morning dose on days 5 and 10 of supplementation, evening dose on day 8, and midday dose on day 12 during the 24 day dosing period due to difficulties in swallowing the capsules. Subject ID# 34 missed the morning dose on day 14. Due to oily diarrhea the period of fish oil supplementation was shortened by 11 days and reduced to 4 capsules Lovaza™ TID (5,580 mg/day EPA and 4500 mg/day DHA) for the last 5 days by subject ID# 34. Similar adverse events in subject ID# 37.1 accounted for the reduction to 4 capsules of Lovaza™ TID after 5 days of protocol-conform fish oil supplementation. The safety laboratory assessments at screening and exit visit were both within normal range for the complete blood count, blood chemistry, lipid and coagulation panels (Supplemental Table 2). Spontaneous blood pressure was on average (across all clinical visits) 120±13 mmHg over 71±10 mmHg (Supplemental Figure 1b); spontaneous heart rate was 71±12 beats/minute (Supplemental Figure 1c); oral temperature was 97.6±0.9 °F. Serious adverse events did not occur. The adverse event profiles are listed in Supplemental Table 6 for the high dose Lovaza fish oils study and in Supplemental Table 7 for the study *"Evoked Endotoxemia"*.

Lipidomics

Specialized pro-resolving mediators (SPMs)

RvD1 and RvE1 were not detected in plasma samples from both clinical studies as evident in the chromatograms of Figure 1b and Figure 2b. For PD1, peaks in plasma were seen which met the

LC and MS/MS criteria for authentic material (chromatograms of Figure 1b and Figure 2b). Baseline PD1 concentrations in plasma amounted to 1.0±0.8 pg/mL and 1.2±0.8 pg/mL which rose to 4.1±2.8 pg/mL and 2.7±1.4 pg/mL after 12 and 24 days, respectively, of supplementation with high doses of fish oil; PD1 dropped to 1.7±1.3 pg/mL and 1.4±0.9 pg/mL at 8 and 13 days after discontinuation of fish oil (Figure 1c left). PD1 concentrations in plasma after 4 g fish oil per day over 8 weeks of 3.1±1.2 pg/mL were not different to baseline PD1 of 3.0±1.5 pg/mL. Acute LPS had no discernable impact on PD1 concentrations in plasma, either in the inflammatory or resolution phases as evident from 2.9±1.0 pg/mL at 2 hours post-LPS, followed by 2.5±1.8, 2.8±2.8, 2.7±1.5, and 2.8±0.4 pg/mL at 6, 12, 24, and 48-72 hours post-LPS, respectively (Figure 2 d top left). Protectin DX (PDX), the isomer of PD1, was present in plasma samples from the evoked endotoxemia study in low picogram per milliliter concentrations, but without apparent modulation by either fish oil or LPS (Supplemental Table 3 and Supplemental Figure 3b). While maresin was consistently below the limit of detection before and after supplementation with high doses of fish oil, plasma samples before and after the administration of LPS showed mean concentrations between 4-6 pg/mL. In detail, 4.2±1.4 pg/mL maresin as baseline before the initiation of fish oil was comparable to 4.3±1.8pg/mL after 8 weeks of supplementation. At 2 hours post-LPS, maresin amounted to 6.3±2.8 pg/mL, followed by 4.2±3.5, 5.0±1.6, 4.5±3.0, and 5.8±3.5 pg/mL at 6, 12, 24, and 48-72 hours post-LPS administration, respectively (Figure 2d bottom). Neither Rvd1, RvE1, maresin nor PD1 were detected in urine from subjects supplemented with high doses of fish oil (chromatograms not shown).

Epoxides

Plasma 17(18)-EpETE and 19(20)-EpDPE, two epoxides formed from eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, respectively, were markedly modulated by the dietary intervention with high doses of fish oils. 17(18)-EpETE increased from baseline values of 0.1±0.1 and 0.2±0.1 ng/mL to 2.0±0.8 (1820±1319%, Figure 1g) and 1.7±1.4 ng/mL (1175±793%, Figure 1g) by days 12 and 24 of supplementation, falling to 0.6±0.4 and 0.4±0.3 ng/mL one and two weeks post intervention. Concentrations in a comparable range were observed for 19(20)-EpDPE: 0.3±0.1 ng/mL as baseline, 1.5±0.7 (411±256%, Figure 1g) and 1.6±1.2 ng/mL (355±227%, Figure 1g) at days 12 and 24 of supplementation, and 0.7±0.4 and 0.6±0.3ng/mL at one and two weeks after discontinuing fish oil. A similar modulation was seen for the EPA-derived epoxide species in plasma, 8(9)-EpETE, 11(12)-EpETE, and 14(15)-EpETE, as well as for the DHA-derived epoxide, 16(17)-EpDPE (Supplemental Table 4). The ω-6 PUFA-derived epoxides in plasma, EETs, were not influenced by fish oil administration (Figure 1, Supplemental Table 4).

Supplementation with low doses of Lovaza fish oils increased the formation of 8(9)-EpETE by 419±265% compared to pre-fish oil baseline, 11(12)-EpETE by 1482±1239%, 14(15)-EpETE by 432±288%, and 17(18)-EpETE by 311±199% (absolute concentrations reported in Supplemental Table 5). Similarly, 16(17)-EpDPE rose by 255±190% and 19(20)-EpDPE by 1375±2181%. Exposure to LPS did not induce directional changes in these epoxides. Neither fish oil nor LPS altered the plasma concentrations of EETs (Supplemental Table 5).

Isoprostanes

The ω-3 PUFA-derived isoprostane, 5-*epi*-8,12-*iso*-iPF$_{3\alpha}$-VI, increased in urine from pre-treatment values of 1.0±0.3 and 0.9±0.4 ng/mg creatinine to 6.1±1.9 and 7.2±2.2 ng/mg creatinine on days 12 and 24 of high dose supplementation, falling back to 2.8±0.8 and 1.5±0.3 ng/mg creatinine at 1 and 2 weeks after discontinuing fish oil (Figure 1e). Urinary 8,12-*iso*-iPF$_{2\alpha}$-VI, a ω-6 PUFA-derived isoprostane, showed no modulation by the intervention; mean concentrations amounted to 3-4 ng/mg creatinine (Figure 1f). The detection of ω-3 PUFA-derived isoprostanes was established in healthy subjects on an *ad libitum* diet in a prior pilot study. Urinary 5-*epi*-8,12-*iso*-iPF$_{3\alpha}$-VI concentrations amounted to 1.3±0.4 ng/mg creatinine, while the well-established ω-6 PUFA-derived isoprostanes, 8,12-*iso*-iPF$_{2\alpha}$-VI and the less abundant iPF$_{2\alpha}$-III reached 7.5±3.1 and 0.7±0.3 ng/mg creatinine, respectively, in a separate cohort of 21 healthy participants (age 28±7.4 years of age; 12 male; 29% AA, 81% CAU).

Eight weeks of supplementation with 4 g/d Lovaza elevated urinary 5-*epi*-8,12-*iso*-iPF$_{3\alpha}$-VI from 0.7±0.4 to 3.5±2.3 ng/mg creatinine. At 1 hour post-LPS, 5-*epi*-8,12-*iso*-iPF$_{3\alpha}$-VI amounted to 3.3±1.5 ng/mg creatinine, followed by 3.9±1.0, 6.0±5.3, and 2.8±2.2 ng/mg creatinine at 2, 4, and 24 hours post-LPS administration, respectively (Figure 2i left). Concentrations of the ω-6 PUFA-derived 8,12-*iso*-iPF$_{2\alpha}$-VI in urine showed no directional changes; 8.6±4.5 ng/mg creatinine before and 7.5±3.0 ng/mg creatinine after supplementation with fish oil, succeeded by 8.5±4.9, 9.3±3.6, 14.0±10.4, and 6.0±3.1 ng/mg creatinine at 1, 2, 4, and 24 hours post-LPS administration, respectively (Figure 2i right).

Ratios of ω-3 and ω-6 PUFA in red blood cells

The ratios of ω-3 to ω-6 PUFA were assessed in erythrocytes to monitor the compliance with the high dose supplementation regimen. The relationship of EPA/AA rose by one order of magnitude

from pre-treatment 0.012±0.004 and 0.011±0.003 to 0.096±0.031 and 0.113±0.033 by days 12 and 24 of supplementation, falling to 0.073±0.021 and 0.065±0.017 at one and 2 weeks post intervention. The ratio of DHA/AA in erythrocytes increased from 0.108±0.025 and 0.103±0.022 to 0.136±0.024 and 0.158±0.02 on days 12 and 24 on fish oil to 0.169±0.021 for both one and 2 weeks post-fish oil (Figure 1d).

In the endotoxemia study, ω-3 to ω-6 PUFAs were quantified as percentage of the total fatty acid pool in erythrocytes. Here, EPA increased from 0.5±0.1 % at baseline to 2.7±0.6 % after 8 weeks of 4 g/d Lovaza, while DHA rose form 6.0±1.1 % to 9.4±1.7 %. AA changed from 93.5±1.0 % at baseline to 87.0±1.6 % after taking fish oil (Figure 2c)

Markers of Inflammation. Administration of LPS elevated a panel of cytokines and acute phase proteins (Figure 2e-k). The maximum increase in plasma concentrations of TNF-α occurred 2 hours post administration of LPS, from initially 0.8±0.3 pg/mL prior to LPS to 12.1±4.3 pg/mL. CRP rose from 0.6±0.5 pg/mL to 10.5±7.1 pg/mL at 24 hours after LPS; MCP-1 from 142.0±22.9 pg/mL to 1919.6±703.4 pg/mL at 4 hours after LPS; IL-6 from 3.7±2.5 pg/mL to 56.6±34.8 pg/mL at 2 hours after LPS; IL-10 from 0.9±0.3 pg/mL to 15.1±7.5 at 4 hours after LPS; SAA from 4.1±2.4 pg/mL to 74.8±25.7 pg/mL at 24 hours after LPS; and IL-1-RA from 152.1±55.8 pg/mL to 16710±9061 pg/mL at 4 hours post LPS administration (Table 1).

Discussion

Derivatives of fish oils have been implicated in a variety of biological activities (49) including the resolution of inflammation (50). Although the therapeutic efficacy of dietary supplementation with fish oils remains controversial (6, 7), observational studies have correlated diets rich in fish to desirable clinical outcomes (1, 51). Whether this relates to a substitution of dietary saturated fatty acids, a direct effect of fish oils or confounding lifestyle factors associated with a diet rich in fish is unknown. When fish oils, such as EPA and DHA, substitute for dietary AA there is a shift in formation from dienoic prostanoids (e.g. PGE_2) to trienoic compounds (e.g. PGE_3) which may differ in biological properties when applied *in vitro* (52). In recent years, there has been considerable interest in the transcellular formation of bioactive lipids, such as might result from substrate exchange between platelets, neutrophils and endothelial cells (50). In particular, transcellular products formed from fish oils - resolvins, maresins and protectins - have exhibited anti-inflammatory properties when administered *in vitro* or to animal models (53, 54). While such observations highlight the therapeutic potential of synthetic SPMs, their relevance as endogenous mediators of inflammation is more controversial. Interpretation of the literature is constrained by two limitations – the use of nonspecific immunoassays and the extrapolation of assays of cellular capacity to form SPMs, often with manipulation *ex vivo*, to infer actual rates of biosynthesis. Both limitations confounded estimates of prostanoid formation in the past; thus the capacity to form prostaglandins greatly exceeds actual biosynthetic rates (55) and immunoassays often grossly overestimated amounts of these compounds detected in biological fluids by more precise physico-chemical methods (56).

Here we sought evidence for formation of bioactive lipids at baseline in healthy volunteers receiving either a high dose of marine lipids delivered as Lovaza fish oil [21 g/d] known to

modulate blood pressure and platelet function (11, 12) or a dosing regimen [4 g/d] more commonly used in clinical trials designed to seek therapeutic efficacy (57). In the latter case, we further characterized the lipidomic response to LPS, both during the evoked inflammation and during the period of its resolution.

In both studies, supplementation with fish oils shifted the bioactive lipid profile *in vivo*. This was apparent in a supplementation related increase in the ratio of EPA and DHA to AA in erythrocyte membranes and in the endogenous biosynthesis of ω-3 derived products of the epoxygenase enzymes and free radical catalyzed formation of isoprostanes. Dose dependent changes in these compounds were observed, exemplified by the respective 3-fold and 10-fold increase from baseline for the EPA-derived epoxide 18(19)-EpETE at the two doses of fish oils. By contrast, SPMs were either undetectable or when identified in trivial amounts, appeared largely unrelated to the periods or doses of fish oil supplementation. The inflammatory response to LPS administration was reflected by evoked cytokines; SPMs were not altered in response to LPS during either the inflammatory (2-4 hrs post administration) or resolution phases (samples measured out to 48-72 hours) in these healthy volunteers.

In the case of resolvins D1 and E1 in plasma, we utilized deuterated authentic derivatives as internal standards for their detection by mass spectrometry and established lower limits of detection in plasma ranging from 3.4-4.3 pg/ml for resolvin D1 and 7.8-10.4 pg/ml for resolvin E1. By contrast, protectin-1 (PD1) levels were detectable and doubled from ~2 pg/ml to 4 pg/ml in plasma during supplementation with 21 g/d Lovaza and fell after the period of supplementation (Figure 1c). PD1 was detectable also on the lower dose of fish oils but did not alter during the inflammatory or resolution phases post LPS (Figure 2d), suggesting that non-

enzymatic formation of PD1 has to be considered (Supplemental Table 8 and Supplemental Figure 6b). Low concentrations of maresins were also detected, but apparently unaltered by either dietary supplementation or administration of LPS. For our analyses, we have no evidence that signals of the authentic endogenous SPMs might be suppressed by the respective deuterated internal standards (Supplemental Table 9 and Supplementary Figure 6c). SPMs were not detectable in urine samples from subjects with high dose supplementation of fish oils, however, some maresin and RvD1 might be present in its glucuronidated form (Supplemental Tables 10-13 and Supplemental Figure 6e,f).

Few studies have utilized mass spectrometry to assess SPM formation in response to fish oil supplementation in humans (19) and none have assessed their formation during resolution of evoked inflammation in vivo. Average plasma levels of RvE1 0.521 nM (182.6 pg/mL) and of RvD1 of 0.0454 nM (17.1 pg/mL) were reported in a study of healthy volunteers on an ad lib diet (58). By contrast, a randomized and placebo-controlled supplementation of 16 volunteers with 3 g/d fish oil over 10 weeks failed to detect RvD1 and RvE1 in plasma (59). However, labeled authentic standards were not applied in either study.

Although there is a paucity of data relating synthetic SPM administration with attained systemic exposure (60-64) and functional effects in mouse models (65), administration of 100 ng/mouse RvD1 resulted in plasma levels of immunoreactive RvD1 peaking at 850 pg/ml three hours later and descending to 200 pg/ml over the ensuing 24 hours during which leukocyte infiltration in zymosan A-induced acute peritonitis was reduced. While no such data are available using physico-chemical estimates of RvD biosynthesis, assumption of the analytical validity of the

approach in this study infers an anti-inflammatory effect at concentrations greatly in excess of the limits of detection of our analysis.

In conclusion, we have failed to detect RvD1 or RvE1 in the plasma of volunteers administered fish oils or in response to an acute inflammatory stimulus after administration of LPS. While maresins and PD1 were detected at low concentrations they were unaltered during evoked inflammation or its resolution. This was in marked contrast to the formation of epoxygenase and free radical catalyzed products of fish oils. For now, the results of these studies question the relevance of endogenous SPMs to the putative anti-inflammatory effects of fish oils in humans.

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Authors' contributions

The contribution each author made to the manuscript are as follows: literature search (CS, NA, JAL, JFF, MPR, GAF), study design (CS, MPR, GAF), data collection (CS, NA, JFF, GAF), mass spectrometry (CS, NA, JAL, GAF), data analysis (CS, NA, JAL, JFF, MPR, GAF), data interpretation (CS, NA, JAL, JFF, MPR, GAF), writing (CS, JAL, GAF), figures (CS, GAF), and tables (CS, JFF, GAF).

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Figures

Figure 1

(a) Study design *"High doses of fish oils"*. Healthy volunteers, n=12 (7♀, 58%; 30.8±11.6 years of age) were supplemented with 17.6 g/day n-3 PUFAs (7 capsules TID of Lovaza™) for 24.2±2.3 days. Timed blood & urine sampling were collected from two weeks prior until two weeks after the intervention.

(b) Representative chromatograms from plasma samples of a single study participant before and after supplementation with fish oil show signals i) for d2-RvD1 in red and no identifiable authentic RvD1; transitions monitored m/z 375→m/z 141 (RvD1), m/z 377→m/z 141 (d2-RvD1), CE 14 eV; ii) for d4-RvE1 in red and no identifiable authentic RvE1; transitions monitored m/z 349→m/z 161 (RvE1), m/z 353→m/z 162 (d4-RvE1), CE 18 eV; iii) for d2-maresin in red and no identifiable authentic maresin; transitions monitored m/z 359→m/z 177 (maresin), m/z 361→ m/z 179 (d2-maresin), CE 14 eV; and iv) for d2-protectin D1 in red and authentic protectin D1 in blue; transitions monitored m/z 359→m/z 153 (PD1), m/z 361→m/z 153 (d2-PD1), CE 15 eV. Authentic protectin D1 appears in plasma in concentrations close to the level of detection; limitations are however, that wide peaks compromise clear identification and that concentrations are not dose-dependent.

(c) (Left) Mean±SD plasma concentrations of plasma Protectin D1 (PD1) before, during and after supplementation of healthy volunteers (n=12) with high doses of fish oil; the red dotted line represents the approximated limit of detection for PD1. (Right) The relationship of PD1 (ordinate) and plasma 19(20)-EpDPE (abscissa) shows a weak association between the two species derived from DHA; area shaded in grey depicts the confidence curve fit.

(d) Ratios of EPA/AA and DHA/AA in red blood cell membranes before, during and after supplementation with high doses of fish oil.

(e) Urinary 8,12-*iso*-iP$F_{3\alpha}$-VI, derived from EPA and DHA, as percent change from baseline (red dotted line);

(f) Urinary 8,12-*iso*-iP$F_{2\alpha}$-VI, derived from AA, as percent change from baseline (red dotted line). Baseline was averaged from n=3 measurements during the two weeks prior to start of supplementation with fish oil.

(g) Plasma 17(18)-EpETE (left), derived from EPA, and plasma 19(20)-EpDPE (right), derived from DHA, as percent change from baseline (red dotted line).

(h) Several EETs in plasma, derived from AA, as percent change from baseline (red dotted line).

Boxplots indicate median, 25% and 75% quartiles and whiskers drawn to the furthest point within 1.5 x interquartile range (IQR) from the box. Note that the unit range on the ordinates varies considerably.

Figure 2

(a) Study design *"Evoked Endotoxemia"*. Healthy volunteers, n=6 (3♀, 50%; 24.3±4.3 years of age) were supplemented with 3.4 g/day n-3 PUFAs (2 capsules BID of Lovaza™) for 50±6.8 days followed by an intravenous bolus of 0.6 ng/kg body weight bacterial lipopolysaccharide (LPS). Timed blood & urine sampling were collected at enrollment, at the end of supplementation with n-3 PUFAs (corresponding to 'pre-LPS'), and in short intervals during the intervention with LPS.

(b) Representative chromatograms from plasma samples of a single study participant before and at the end of the supplementation period with fish oil as well as 12, 24 and 48-72 hours post intervention with lipopolysaccharide (LPS) show signals i) for d_2-RvD1 in red and no identifiable authentic RvD1; transitions monitored m/z 375→m/z 141 (RvD1), m/z 377→m/z 141 (d_2-RvD1), CE 14 eV; ii) for d_4-RvE1 in red and no identifiable authentic RvE1; transitions monitored m/z 349→m/z 161 (RvE1), m/z 353→m/z 162 (d_4-RvE1), CE 18 eV; iii) for d_2-maresin in red and authentic maresin in blue; transitions monitored m/z 359→m/z 177 (maresin), m/z 361→ m/z 179 (d_2-maresin), CE 14 eV; and iv) for d_2-PD1 in red and authentic PD1 in white and with earlier retention time than its isomer PDX marked in blue; transitions monitored m/z 359→m/z 153 (PD1), m/z 361→m/z 153 (d_2-PD1), CE 15 eV. Authentic maresin and protectin D1 appear in plasma in concentrations close to the level of detection; limitations are, however, that wide peaks compromise clear identification and that concentrations are not dose-dependent. Chromatograms not shown for time points 2 and 6 hours post LPS.

(c) EPA and DHA percentage (left) and AA percentage (right) of total fatty acids in red blood cell membranes before and after supplementation with fish oil.

(d) Plasma concentrations of Protectin D1 (PD1) (Top Left) and Maresin (Bottom Left) prior to and after conditioning with a clinical dose of fish oil (4 g/d), and subsequent acute challenge with LPS. The relationship of PD1 (ordinate) and 19(20)-EpDPE (abscissa) shows a weak association between the two species derived from DHA; area shaded in grey depicts the confidence curve fit (Top Right). Formation of Maresin, also derived from DHA, is not associated with 19(20)-EpDPE (Bottom Right).

(e, f, g, h, i, j & k) The time course of (e) TNF-α, (f) CRP, (g) MCP-1, (h) IL-6, (i) IL-10, (j) SAA, and (k) IL1-RA in plasma is shown as percent change from baseline, i.e. pre-fish oil

measurements. Note that time point zero (0) denotes the condition pre-LPS which coincides with day 50±6.8 of supplementing the healthy volunteers with 2 capsules Lovaza™ BID.

(l) (left) Urinary 8,12-*iso*-iPF$_{3\alpha}$-VI, derived from EPA and DHA, as percent change from baseline (pre-fish oil measurements, red dotted line); (right) urinary 8,12-*iso*-iPF$_{2\alpha}$-VI, derived from AA, as percent change from baseline (pre-fish oil measurements, red dotted line). Note that time point zero (0) denotes the condition pre-LPS which coincides with day 50±6.8 of supplementing the healthy volunteers with 2 capsules Lovaza™ BID.

(m) Plasma concentrations in pg/mL of EETs formed from AA (left), EpETEs derived from EPA (center), and EpDPEs formed from DHA (right) before and after 8 weeks of supplementing with 2 capsules Lovaza™ BID.

Boxplots indicate median, 25% and 75% quantiles and whiskers drawn to the furthest point within 1.5 x interquartile range (IQR) from the box. Note that the unit range on the ordinates varies considerably.

Tables

Table 1. Markers of Inflammation

Human Study "Evoked Endotoxemia"	**Unit**	**Pre-fish oil**	**0 hrs**	**1 hrs post-LPS**	**2 hrs post-LPS**	**4 hrs post-LPS**	**6 hrs post-LPS**	**12 hrs post-LPS**	**24 hrs post-LPS**
			After ≈8 weeks of 4 g/d fish oil						
TNF-α	pg/mL	1.1±0.4	0.8±0.3	4.9±4.1	12.1±4.3	-	3.1±0.9	1.7±0.5	-
	%	0.0	-15.5±27.4	463.9±533.4	1213±670	-	214.9±65.5	64.5±18.8	-
CRP	mg/L	0.5±0.5	0.6±0.5	-	-	-	1.2±0.8	5.5±1.5	10.5±7.1
	%	0.0	10.7±45.6	-	-	-	214.5±128.5	1551.7±771.7	3336.4±2890.2
MCP-1	pg/mL	148.3±26.9	142.0±22.9	122.7±30.4	684.2±428.2	1919.6±703.4	373.7±151.8	133.2±25.2	-
	%	0.0	-3.0±14.0	401.6±425.2	1160±342.7	146.0±83.7	-16.7±16.7	-7.5±24.0	-
IL-6	pg/mL	1.1±0.5	3.7±2.5	5.0±2.9	56.6±34.8	27.2±11.6	11.1±8.9	9.1±8.7	-
	%	0.0	121.9±163.1	2458.4±1885.3	2318.8±1485.2	805.4±795.2	217.1±286.8	268.8±331.0	-
IL-10	pg/mL	0.9±0.4	0.9±0.3	1.6±1.3	9.1±6.4	15.1±7.5	4.5±1.7	1.7±0.9	-
	%	0.0	6.5±27.6	57.8±93.0	892.4±696.5	1699.2±1079.2	413.7±222.0	89.4±109.8	-
SAA	mg/L	4.3±2.7	4.1±2.4	-	-	-	16.4±6.9	44.7±16.7	74.8±25.7
	%	0.0	0.2±24.0	-	-	-	326.0±171.1	1048.3±332.6	1817.2±458.4
IL-1-RA	pg/mL	150.3±44.8	152.1±55.8	150.2±56.1	261.0±118.3	16710±9061	5171±2212	880.1±257.3	-
	%	0.0	3.0±35.7	-0.37±22.5	75.0±58.8	12076±8047	3740±2198	527.0±271.4	-

Figure 1

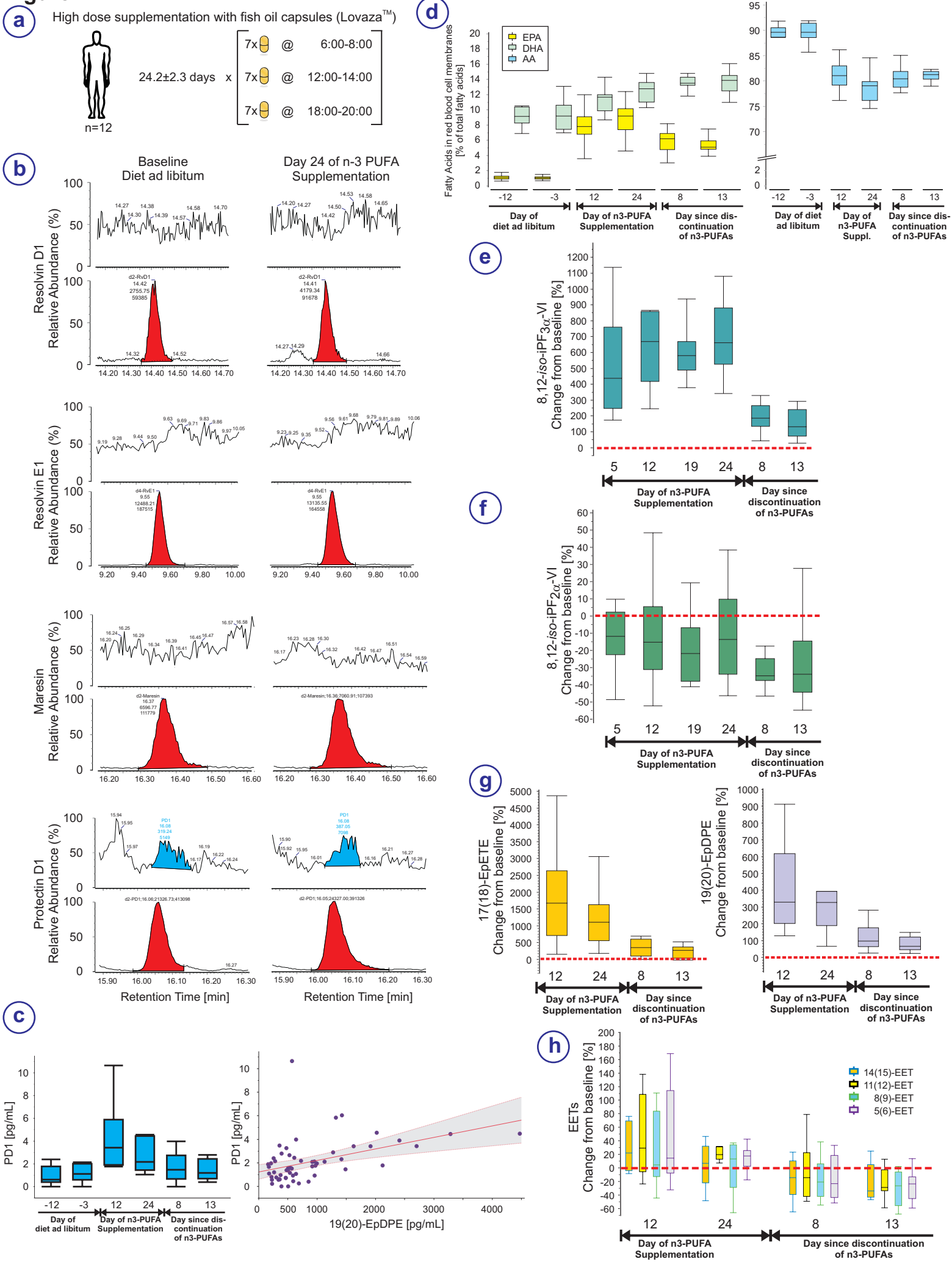

a
High dose supplementation with fish oil capsules (Lovaza™)
n=12
24.2±2.3 days x
7x @ 6:00-8:00
7x @ 12:00-14:00
7x @ 18:00-20:00
b
Baseline
Diet ad libitum
Day 24 of n-3 PUFA
Supplementation
Resolvin D1
Relative Abundance (%)
Resolvin E1
Relative Abundance (%)
Maresin
Relative Abundance (%)
Protectin D1
Relative Abundance (%)
Retention Time [min]
Retention Time [min]
c
PD1 [pg/mL]
Day of diet ad libitum
Day of n3-PUFA Supplementation
Day since dis-continuation of n3-PUFAs
PD1 [pg/mL]
19(20)-EpDPE [pg/mL]
d
Fatty Acids in red blood cell membranes [% of total fatty acids]
EPA
DHA
AA
Day of diet ad libitum
Day of n3-PUFA Supplementation
Day since dis-continuation of n3-PUFAs
Day of diet ad libitum
Day of n3-PUFA Suppl.
Day since dis-continuation of n3-PUFAs
e
8,12-iso-iPF3α-VI
Change from baseline [%]
Day of n3-PUFA Supplementation
Day since discontinuation of n3-PUFAs
f
8,12-iso-iPF2α-VI
Change from baseline [%]
Day of n3-PUFA Supplementation
Day since discontinuation of n3-PUFAs
g
17(18)-EpETE
Change from baseline [%]
19(20)-EpDPE
Change from baseline [%]
Day of n3-PUFA Supplementation
Day since discontinuation of n3-PUFAs
Day of n3-PUFA Supplementation
Day since discontinuation of n3-PUFAs
h
EETs
Change from baseline [%]
14(15)-EET
11(12)-EET
8(9)-EET
5(6)-EET
Day of n3-PUFA Supplementation
Day since discontinuation of n3-PUFAs

Figure 2

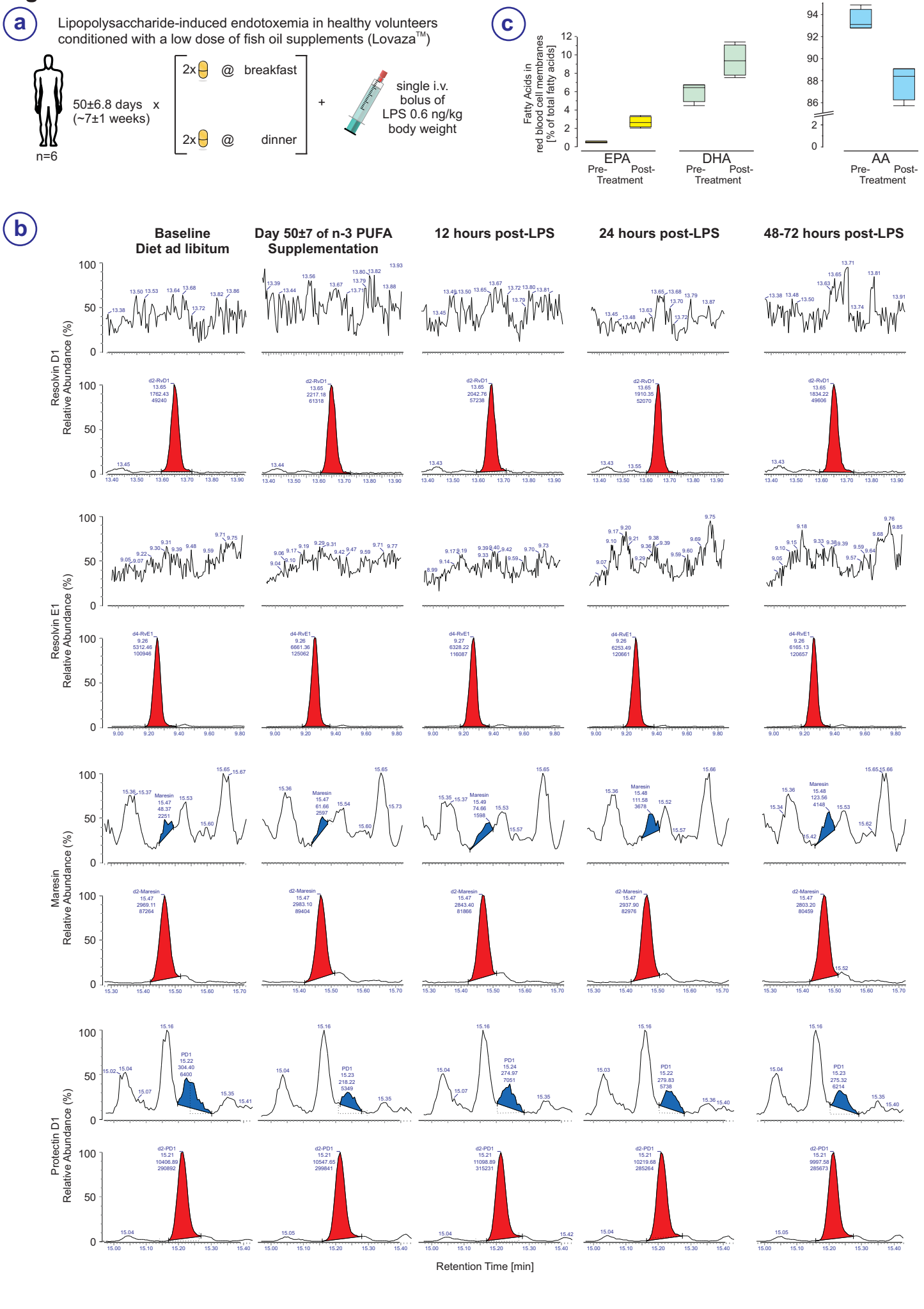
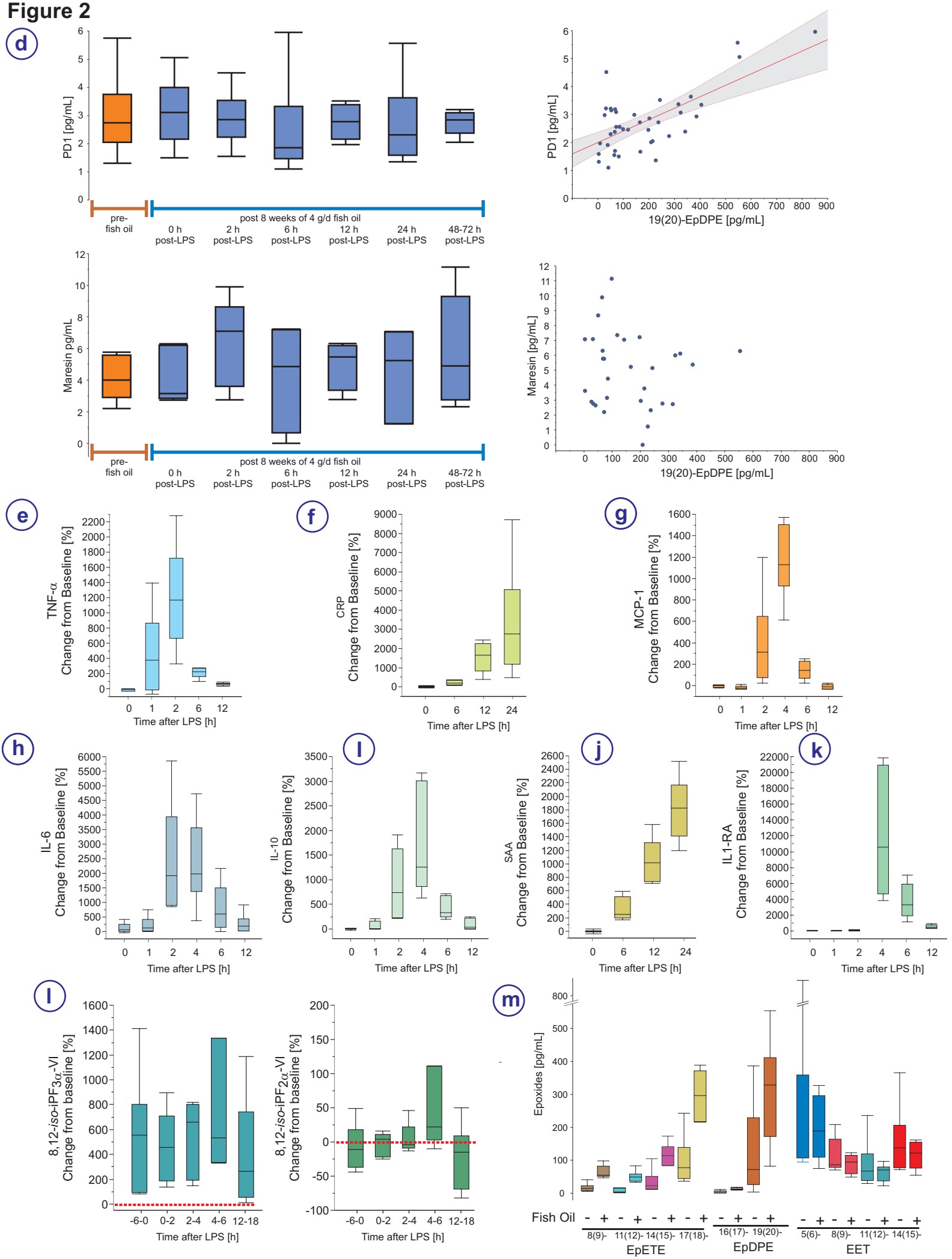

a
Lipopolysaccharide-induced endotoxemia in healthy volunteers conditioned with a low dose of fish oil supplements (Lovaza™)
n=6
50±6.8 days x (~7±1 weeks)
2x @ breakfast
2x @ dinner
+
single i.v. bolus of LPS 0.6 ng/kg body weight
c
Fatty Acids in red blood cell membranes [% of total fatty acids]
EPA
DHA
AA
Pre-Treatment
Post-Treatment
b
Baseline Diet ad libitum
Day 50±7 of n-3 PUFA Supplementation
12 hours post-LPS
24 hours post-LPS
48-72 hours post-LPS
Resolvin D1 Relative Abundance (%)
Resolvin E1 Relative Abundance (%)
Maresin Relative Abundance (%)
Protectin D1 Relative Abundance (%)
d2-RvD1
d4-RvE1
d2-Maresin
d2-PD1
Retention Time [min]

Figure 2


d
PD1 [pg/mL]
pre-fish oil
post 8 weeks of 4 g/d fish oil
0 h post-LPS
2 h post-LPS
6 h post-LPS
12 h post-LPS
24 h post-LPS
48-72 h post-LPS
19(20)-EpDPE [pg/mL]
Maresin pg/mL
Maresin [pg/mL]
e
TNF-α
Change from Baseline [%]
Time after LPS [h]
f
CRP
g
MCP-1
h
IL-6
i
IL-10
j
SAA
k
IL1-RA
l
8,12-iso-iPF3α-VI
8,12-iso-iPF2α-VI
m
Epoxides [pg/mL]
Fish Oil
8(9)-
11(12)-
14(15)-
17(18)-
EpETE
16(17)-
19(20)-
EpDPE
5(6)-
8(9)-
11(12)-
14(15)-
EET