Serum non-esterified fatty acids have utility as dietary biomarkers of fat intake from fish, fish oil and dairy in women

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Running title:

Serum non-esterified fatty acids as biomarkers of dietary fat

Abbreviations:

DHA, docosahexaenoic acid; DQI; diet quality index; EPA, eicosapentaenoic acid; FO, fish oil; FFQ, food frequency questionnaire; MSI-NACE-MS, multisegment injection-non-aqueous-capillary electrophoresis–mass spectrometry; NEFA, non-esterified fatty acids; OCFA, odd-chain fatty acid; ω-3 PUFA, omega-3 polyunsaturated fatty acid.

ABSTRACT

Nutritional studies rely on various biological specimens for fatty acid (FA) determination, yet it is unclear how levels of serum non-esterified FA (NEFAs) correlate with other circulating lipid pools. Here, we used a high throughput method (< 4 min/sample) based on multisegment injection-non-aqueous-capillary electrophoresis–mass spectrometry (MSI-NACE-MS) to investigate whether specific serum NEFAs have utility as biomarkers of dietary fat intake in women. We first identified circulating NEFAs correlated with long-term/habitual food intake among pregnant women with contrasting dietary patterns (n = 50). Acute changes in serum NEFA trajectories were also studied in non-pregnant women (n = 18) following high-dose (5 g/day) fish oil (FO) supplementation or isoenergetic sunflower oil placebo over 56 days. In the cross-sectional study, serum omega-3 (ω-3) FA correlated with self-reported total ω-3 daily intake, notably eicosapentaenoic acid (EPA) as its NEFA (r = 0.46; p = 0.001), whereas pentadecanoic acid was associated with full-fat dairy intake (r = 0.43; p = 0.002), outcomes consistent with results from total FA serum hydrolysates. In the intervention cohort, serum ω-3 NEFAs increased 2.5-fold from baseline within 28 days following FO supplementation, and this increase was most pronounced for EPA (p = 0.0004). Unlike for docosahexaenoic acid, circulating EPA as its NEFA also strongly correlated to EPA concentrations measured from erythrocyte phospholipid hydrolysates (r = 0.66; $p = 4.6 \times 10^{-10}$), and was better suited to detect dietary non-adherence. We conclude that MSI-NACE-MS offers a rapid method to quantify serum NEFAs and objectively monitor dietary fat intake in women that is complementary to diet records or food frequency questionnaires.

Keywords: fatty acid metabolism, omega-3 fatty acids, nutrition, mass spectrometry, capillary electrophoresis, dietary fat, high-throughput assay, pregnancy, maternal health, lipid metabolism.

INTRODUCTION

Accurate assessment of dietary fat intake remains a methodological challenge, reflecting decades of conflicting evidence regarding the benefits of a low-fat diet for public health (1). Validated semi-quantitative food frequency questionnaires (FFQ) are widely used dietary assessment tools in large-scale observational studies as they can reliably differentiate habitual dietary patterns, as well as estimate micro- and macronutrient intake in a cost-effective manner (2). However, FFQ are prone to recall bias, errors in estimation of true portion sizes, as well as selective reporting (3); this problem is exacerbated when assessing habitual fat intake due to the large variation of fatty acid (FA) species in the diet, and the tendency for underreporting fat consumption (4). Errors associated with participant self-reporting have been recognized as one of the greatest obstacles in nutritional epidemiology, limiting our ability to capture food exposures in contemporary societies (5). Comprehensive metabolite profiling (*i.e.,* metabolomics) offers a strategy to objectively measure complex dietary patterns, including the discovery of new biomarkers of recent food intake (6). An optimal dietary biomarker is readily measurable in a minimally invasive human biofluid (urine or blood), specific to a single food group (selective), responsive to changes in the amount of food consumed (dose-response) over a desired time frame (time-response), and is ideally not generated *in vivo* nor extensively biotransformed (exogenous) (7). For example, proline betaine is a reliable dietary biomarker of recent citrus intake (< 24 h) in plasma and urine samples that has been validated in several independent observational and intervention studies (3, 8-10). Such biomarkers generally do not exist for most FAs since they are synthesized *de novo* from carbohydrates and other FA precursors; however, there are some exceptions, such as certain polyunsaturated FAs (PUFAs), odd-chain FA (OCFAs), and *trans*-FA (TFAs), which are primarily derived from specific food sources and thus largely not synthesized *in-vivo* (11).

There is an evolving consensus regarding optimal nutritional guidelines for dietary fat with greater emphasis placed on assessing the specific type of fat within complex dietary patterns as opposed to measuring total fat intake and single nutrients (12). For example, many jurisdictions have now

restricted industrial TFA exposures from processed foods due to their deleterious effects on cardiovascular health as compared to non-restricted populations (13). Recent findings from the 21-country Prospective Urban and Rural Epidemiological (PURE) study reported that total fat is correlated with lower total mortality, and increased saturated fat or total dairy consumption with lower risk for cardiovascular events (14, 15); however, these studies have relied on estimating intakes of FAs from FFQ, and gathered health information from different countries with the potential for residual confounding. As a result, high throughput methods for objective measurements of circulating FA concentrations are thus needed to provide a more standardized approach for accurate assessment of habitual fat consumption. This is important given conflicting data regarding the putative health benefits of dietary intake of essential omega-3 (ω -3) PUFAs (16), such as clinical trials involving supplementation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil (FO) or a prescription based EPA analog for primary or secondary prevention of cardiovascular disease events (17, 18). Various biospecimen types have been used for FA determination and each reflects a different time interval associated with dietary fat intake, including adipose tissue (1-2 years), erythrocyte membrane (2-3 months), serum phospholipids (PL) or cholesterylesters (CE) (past few days), and triglycerides (TG) (past few hours) fractions (4, 11, 19). While adipose tissue may be useful for long-term assessment of dietary fat intake, such biopsies are invasive and cannot be routinely collected. Also, erythrocytes isolated from whole blood are prone to hemolysis during processing and long-term storage (20, 21) and they are not often available in most bio-bank repositories unlike serum or plasma. As of 2016, it was reported that 90% of studies analyzing ω-3 PUFA used erythrocytes membrane PL, serum PL, or total (hydrolyzed) plasma lipids (22). Alternatively, fasting serum non-esterified FA (NEFAs) are a more accessible protein-bound lipid pool, which are released into circulation by the hydrolyzing action of lipases on TG from adipose tissue (4). Nevertheless, there have been few reports to date examining the utility of NEFAs as biomarkers of dietary intake in nutritional studies as compared to total FA from serum extracts, and other blood fractions or adipose tissue samples (19, 23). For instance, gas chromatography (GC) methods allow for high efficiency separation of FA and their isomers (24), but are limited by long analysis times and pre-column chemical

derivatization procedures that contribute to bias due to hydrolysis of esterified FA from other lipid classes, impeding reliable serum NEFA determination (25).

Herein, we performed two studies to assess the utility of serum NEFAs as convenient dietary biomarkers of fat intake in women when using multisegment injection-non-aqueous-capillary electrophoresis–mass spectrometry (MSI-NACE-MS). This multiplexed separation method offers higher sample throughput (< 4 min/sample) and stringent quality control (QC) for direct analysis of NEFAs from serum extracts without fractionation, hydrolysis and pre-column chemical derivatization unlike conventional GC methods (26). For the first time, we assess a cross-section of pregnant women with contrasting diet quality patterns (27) when using MSI-NACE-MS to identify specific serum NEFAs that serve as biomarkers of habitual intake of fish/seafood, full-fat dairy products, and fiber as compared to FAs from total serum hydrolysates. Also, time-dependent changes in serum eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in young women participating in a placebo-controlled, repeated-measures trial of high-dose fish oil (FO) supplementation (28) were also examined as their circulating NEFAs relative to independent erythrocyte membrane PL measurements.

MATERIALS AND METHODS

Serum NEFA Biomarkers of Dietary Fat Intake in Pregnant Women from FAMILY

The Family Atherosclerosis Monitoring In earLY life (FAMILY) study is a prospective birth cohort study involving 839 predominantly white European pregnant women recruited from the greater Hamilton area between 2002 and 2009 (27). Fasting blood samples were collected in the second trimester, and serum was fractionated within 2 h from collection according to standard protocols and stored at -80 °C. Comprehensive clinical and dietary data from all participants were also collected. Ethical approval and informed consent from all study participants were obtained. In this study, we used purposive sampling, after exclusion of smokers and women with gestational diabetes mellitus, to generate a subset of women (n = 50) from 226 eligible participants, half of whom consumed a healthy diet and half of

whom consumed a poor quality diet as assessed by a diet quality index (DQI) score (29) with a mean age of 32 years (range of 17 to 43 years) and pre-pregnancy BMI of 27 kg/m^2 (range of 18 to 50 kg/m^2). Briefly, a semi-quantitative FFQ developed for the Study of Health and Risk in Ethnic Groups (SHARE) study (30) was used to assess maternal dietary intake on one occasion after recruitment at about mid-pregnancy by asking the participants to answer questions related to their usual eating habits during the last one year period. The complete FFQ was analyzed by using a database linked to the Canadian Nutrient File. Nutrient composition was calculated as previously described (31), excluding records where the FFQ was 50% incomplete, or with implausible dietary intakes (< 500 or > 4500 kcal/d). Use of supplements was also assessed as part of the FFQ, which included a separate supplemental questionnaire for cod liver or halibut oil supplement usage. The DQI score used to classify the nutritional status of pregnant women into healthy and unhealthy eating categories was based on reported daily servings of foods from 36 harmonized food groups as described previously (29). This aggregate score reflects differences in the overall nutritional quality of foods consumed, which was based on the sum of the daily number of servings of healthy and nutrient-rich foods (*e.g.,* fermented dairy, fish and seafood, vegetables, fruits, whole grains, nuts and seeds) minus the daily number of servings of unhealthy and processed foods (*e.g.,* processed meats, refined grains, fries, snacks, sweets and sweet drinks). A positive DQI score signifies consumption of greater amounts of healthy than unhealthy foods, and vice versa for a negative DQI score. In this study, 25 participants were selected from the top 10th percentile to form the "good diet" group, and the bottom 10th percentile of the cohort comprised the "poor diet" group as summarized in **Figure S1**.

High Dose ω-3 PUFA Supplementation in Women and Serum NEFA Trajectories

Serum NEFA were analyzed using fasting serum samples from a clinical intervention trial in 2017 and 2018 investigating the effect of ω-3 PUFA supplementation from fish oil (FO) on attenuating skeletal muscle atrophy following leg immobilization (28). The trial was registered at the U.S. National Library of Medicine (https://clinicaltrials.gov/) as NCT03059836. Briefly, a cohort of healthy young women with a mean age of 22 years (range of 19 to 31 years) and BMI of 24 kg/m^2 (range of 18 to 26

kg/m^2), were recruited from the greater Hamilton area to participate in a randomized, double-blinded, placebo-controlled intervention study. Participants received either the active treatment arm of a high-dose ω-3 PUFA from FO (3.0 g EPA, and 2.0 g DHA daily, n = 9), or a control based on an isoenergetic and volume equivalent sunflower oil daily (n = 9). Serum samples in the resting fasted state was collected from participants at baseline, and at 28, 42 and 56 days following initiation of the intervention, which were then stored at -80 °C. Participants taking FO supplements within 6 months of the study were excluded, 2 participants had missing residual serum samples from the original study, and 1 participant was reported to be non-compliant to leg immobilization intervention (28), and possibly dietary adherence. Comprehensive analysis of serum NEFAs in this work was compared to FA concentrations reported for the erythrocyte membrane PL fraction measured using a validated protocol based on GC-FID (28).

Validated Method Protocol for Serum NEFA and Total FA Analysis by MSI-NACE-MS

NEFAs from fasting serum samples collected from young women in both studies, along with standard human serum (Sigma S7023) used as a quality control (QC) specimen, were analyzed using a previously validated protocol for rapid serum NEFA determination based on MSI-NACE-MS that included an extensive inter-method comparison to GC-MS (26). In all cases, frozen serum aliquots were thawed once slowly on ice prior to analysis. Briefly, protein-bound circulating FA were extracted from serum in acidified (3.7 % *vol* of 1.0 M HCl) methyl-*tert*-butyl ether (MTBE):methanol (5:1, *vol*) containing butylated hydroxytoluene (BHT, 0.01% *vol*) as an antioxidant additive during sample processing, and a deuterated analog of myristic acid, 14:0-d27 as a recovery standard. Following vigorous shaking, phase separation was then induced by addition of deionized water, and samples were then centrifuged to sediment protein at bottom of vial (at 3000 *g* at 4 °C for 30 min) followed by a biphasic water and ether (top) layer. A fixed volume (200 µL) was collected from the upper MTBE layer into a new vial then dried under a gentle stream of nitrogen gas at room temperature. Dried serum extracts were then stored at -80 °C and at time of analysis reconstituted in 25 µL of acetonitrile/isopropanol/water (70:20:10) with 10 mM ammonium acetate and 50 µM deuterated stearic acid, 18:0-d35 as an internal

standard. For analysis of total (hydrolyzed) serum FAs, acid-catalyzed hydrolysis was performed using 2.5 M sulfuric acid and 0.01% *vol* BHT in toluene followed by incubation at 80 °C for 1 h (26). MTBE extraction was then carried out to recover total serum FA similar to the protocol outlined for serum NEFA. An Agilent 6230 time-of-flight (TOF) mass spectrometer with a coaxial sheath liquid electrospray (ESI) ionization source equipped with an Agilent G7100A CE unit was used for all experiments (Agilent Technologies Inc., Mississauga, ON, Canada). An Agilent 1260 Infinity isocratic pump was used to deliver an 80% *vol* methanol with 0.5% *vol* ammonium hydroxide at a flow rate of 10 µL/min using a CE-MS coaxial sheath liquid interface kit. Separations were performed on bare fused-silica capillaries with 50 µm internal diameter, 360 µm outer diameter and 95 cm total length (Polymicro Technologies Inc., AZ, USA). The applied voltage was set to 30 kV at 25 °C for CE separations together with an isocratic pressure of 20 mbar (2 kPa). The background electrolyte (BGE) was 35 mM ammonium acetate in 70% *vol* acetonitrile, 15% *vol* MeOH, 5% *vol* isopropanol with an apparent pH of 9.5 adjusted by addition of 12% *vol* of ammonium hydroxide. Serum extracts were injected hydrodynamically at 50 mbar (5 kPa) alternating between 5 s for each sample plug and 40 s for the BGE spacer plug for a total of 7 discrete samples analyzed within 30 min for a single run (26, 32). Repeat QC samples introduced in a randomized position for each MSI-NACE-MS run were analyzed for NEFA (n = 8) and total FA analysis (n = 8) for assessment of technical precision of the method. All FA extracts were analyzed directly by MSI-NACE-MS without chemical derivatization when using negative ion mode detection at 3500 V with full-scan data acquisition (*m/z* 50-1700), which allows for comprehensive screening of 24 FA consistently measured in human serum extracts.

Data Processing and Statistical Analyses

MSI-NACE-MS data was analyzed using Agilent Mass Hunter Workstation software (Qualitative Analysis, version B.06.00, Agilent Technologies, 2012). Molecular features were extracted in profile mode within a 10 ppm mass window, and serum NEFAs were annotated based on their characteristic accurate mass (*m/z*) corresponding to their intact deprotonated molecular ion $[M\text{-}H]^-$, and relative

migration time (RMT) reflecting the electrophoretic mobility for anionic FA. Extracted ion electropherograms (EIE) were integrated after smoothing using a quadratic/cubic Savitzky-Golay function (15 points), and integrated peak areas and apparent migration times were normalized to stearic acid-d35 for determination of relative peak area (RPA) and RMT. Least-squares linear regression analysis for external calibration curves and control charts were performed using Excel (Microsoft Office, Redmond, WA, USA). Principal component analysis (PCA) was used for data visualization (*i.e.,* data trends/outlier detection) when comparing the technical variance of QC samples as compared to the overall biological variance of serum NEFA concentrations between-subjects when using MetaboAnalyst 4.0 (33). For multivariate analysis for data visualization and univariate analysis comparing maternal diet sub-groups, data was normalized using a generalized *log* transformation and autoscaled with FDR correction applied for multiple hypothesis testing. Also, a QC based batch-correction algorithm was performed to correct for long-term signal drift in ESI-MS for robust serum NEFA determination using an algorithm available in the *R* Project for statistical computing (34). Normality tests, Pearson and Spearman rank correlations, student's t-test, and nonparametric statistical analysis (Mann-Whitney U test) were performed using the Statistical Package for the Social Science (SPSS, version 18), whereas MedCalc version 12.5.0 (MedCalc Software, Ostend, Belgium) was used for generation of boxplots. To assess the validity of serum NEFAs and total FAs against reported dietary intakes of fish, full-fat dairy, and total fiber, as well as the diet quality index score, we used a Spearman's rank correlation coefficient (r) for non-transformed data. A correlation coefficient of $r = 0.1$ to 0.3 was considered a small effect, $r = 0.3$ to 0.5 a moderate effect, and $r > 0.5$ a large effect. In order to minimize error by accounting for EPA and DHA sources from both the diet and supplementation, a total daily ω-3 PUFA servings score (total ω-3) was devised, calculated from FFQ as the sum of EPA and DHA from self-reported dietary intake (g/day), as well as self-reported supplement use (g/day). Multiple regression models were constructed for *log*-transformed measured serum NEFA concentrations to account for potential confounding variables (*i.e.,* BMI, cholesterol and HDL) that were different ($p < 0.05$) between the two dietary sub-groups. Circulating NEFAs are reported in terms of absolute molar concentrations (μM) as a standardized way to enable data comparisons

independent of analytical platform, sample workup protocols, and total number of FAs measured. A 2-way between and within mixed-model ANOVA (treatment; time) was used for assessing the impact of high-dose FO supplementation to alter circulating NEFA concentrations in healthy/non-pregnant women as compared to a placebo control. A Pearson correlation coefficient for non-transformed data was used to test the association between serum NEFAs and FAs from hydrolyzed erythrocyte PL fraction.

RESULTS

High throughput Serum NEFA Determination by MSI-NACE-MS

Overall, 24 serum FAs (ranging from 9:0 to 24:1) were reliably measured as their NEFAs and/or total hydrolyzed FAs by MSI-NACE-MS from serum ether extracts (> 95%) with acceptable technical precision (CV < 15%) when using standard serum for quality control (QC) as summarized in **Table S1**. Serum FAs were analyzed after normalization of their ion responses to a single deuterated internal standard (18:0-d35) added to all samples, and most circulating FAs were quantified in terms of their absolute concentration (μM) using an external calibration curve. Each run consisted of a serial injection of 6 randomized serum samples together with a QC as shown in **Figure 1A** for representative serum NEFAs annotated by their accurate mass and relative migration time (*m/z*:RMT). Anionic FAs are resolved based on differences in their electrophoretic mobility (*i.e.,* carbon-chain length, degrees of unsaturation) that migrate after the electroosmotic flow (EOF) away from major neutral/zwitter-ionic lipids (*i.e.,* triacylglycerides, phospholipids, cholesterol) when using an alkaline non-aqueous buffer system, and detected as their intact molecular ion [M-H$^-$] under negative ion mode using a coaxial sheath liquid interface (26); however, geometric isomers for certain FAs are not baseline resolved. **Figure 1B** highlights that larger biological variance was evident for fasting serum NEFAs (mean CV = 62%) as compared to corresponding total FAs from serum lipid hydrolysates (mean CV = 32%) in pregnant women (n = 50). Also, good technical precision was confirmed based on a repeat analysis of QC samples (mean CV ≈ 12%, n = 8), and a control chart for a recovery standard (14:0-d27) demonstrated reliable

long-term performance with few samples (≈ 2.6%, n = 114) exceeding warning limits (± 2s). Previous method validation studies demonstrated good mutual agreement for serum FA determination when using MSI-CE-MS as compared to GC-MS (26), which is optimal for higher throughput NEFA screening (< 4 min/sample) without pre-column chemical derivatization, lipid fractionation and/or hydrolysis artifacts as heating is avoided during sample workup. Only about 5-6% of total PUFAs are protein-bound NEFAs (*e.g.,* DHA, EPA and arachidonic acid) unlike other FAs that are not extensively esterified into blood lipids, such as lauric acid (12:0) and myristic acid (14:0) as summarized in **Table S1**.

Serum ω-3 PUFA Status Reflects Differences in Diet Quality and Habitual Fish Intake

Anthropometric and clinical data from second trimester pregnant women classified by their contrasting habitual diets from FFQ are summarized in **Table 1**. Overall, 50 women were selected from eligible FAMILY participants (**Figure S1**) reflecting healthy (median DQI score = 12.0 ± 1.9) and non-healthy (median DQI score = - 9.1 ± 3.0) maternal eating patterns, respectively. In this study, associations of serum NEFA concentrations to self-reported dietary intake from a standardized FFQ were evaluated rather than maternal health or birth outcomes. As expected, pre-pregnancy BMI was lower in the healthy eating diet sub-group, but there were no differences in age, and weight gain during pregnancy, as well as fasting glucose concentrations, hemoglobulin glycation, serum triglycerides, and LDL; however, total and HDL cholesterol were modestly lower in the poor diet quality maternal group (p < 0.02). Importantly, total fiber intake, and daily fish/seafood servings were significantly higher in the healthy eating diet group (p < 0.0001) unlike full-fat dairy intake since it was not used a variable in the DQI score for participant selection. As expected, pregnant women consuming a healthy diet had consistently higher circulating concentrations of ω-3 PUFA, namely DHA and EPA in terms of their serum NEFA and total FA as compared to the poor diet quality maternal sub-group (**Figure 2A and 2B**). Moreover, moderate correlations (r = 0.3 to 0.5; p < 0.05) were measured between serum EPA, DHA, and their sum [EPA+DHA], relative to the DQI score, as well as total ω-3 PUFAs from FFQ based on daily average intakes of EPA and DHA estimated during pregnancy from both dietary sources and FO supplement use

as highlighted in **Table 2**. In fact, the strongest correlation was serum EPA as its NEFA or total FA with self-reported total ω-3 PUFA with $r = 0.46$ and 0.50 ($p < 0.001$), respectively. As for the correlation of circulating DHA with total ω-3 PUFA intake, it was found to be higher for NEFA ($r = 0.40$; $p = 0.0040$) as compared to total serum FA hydrolysate ($r = 0.33$; $p = 0.024$). Only 4 of 50 women were reported to be taking FO supplements during pregnancy, and these women had the highest circulating NEFA concentrations for EPA. **Tables S2 and S3** summarize results from the linear regression model based on measured EPA and DHA concentrations as a function of the DQI score, and total ω-3 PUFA with adjustments for covariates between both diet groups, namely BMI, total cholesterol and HDL. Overall, correlations remained significant ($p < 0.05$) after adjustments for BMI and total cholesterol, as well as HDL in most cases. The scatter plot in **Figure 2C** illustrates the positive correlation ($r = 0.43$, $p = 0.0020$) between measured concentrations for [EPA+DHA] as compared to self-reported ω-3 PUFA intake (g/day), which also includes a histogram for circulating EPA as its NEFA which had a median serum concentration of 1.64 μM. A correlation analysis (**Table S4**) between serum NEFA and total FA also demonstrated a much stronger association for EPA ($r = 0.57$; $p = 2.0 \times 10^{-5}$) as compared to DHA ($r = 0.29$; $p = 0.049$) highlighting the unique attribute of this low abundance circulating ω-3 PUFA.

Serum Odd-chain/Saturated FA Reflect Full-fat Dairy and Total Fiber Intake

Certain SFAs as their serum NEFAs and/or total FA hydrolysates were correlated with self-reported intake of full-fat dairy (**Table 3**). For instance, serum pentadecanoic acid (15:0) as its NEFA had the strongest association to full-fat dairy intake ($r = 0.43$; $p = 0.0020$), whereas heptadecanoic acid (17:0) was not significant ($r = 0.21$; $p = 0.15$). However, 17:0 from total hydrolyzed serum had a weak correlation to full-fat dairy ($r = 0.29$; $p = 0.043$). In this case, total 17:0 was also associated with daily fiber servings ($r = 0.29$; $p = 0.050$), including both soluble ($r = 0.38$; $p = 0.008$), and insoluble fiber ($r = 0.31$; $p = 0.034$) fractions. Also, serum myristic acid (14:0) showed a similar outcome as 15:0 as its NEFA ($r = 0.30$; $p = 0.034$) and total FA ($r = 0.35$; $p = 0.016$) albeit with a more moderate correlation to full-fat dairy intake. The scatter plot in **Figure 2D** highlights the positive correlation of fasting serum

15:0 as its NEFA to self-reported daily intake of dairy products (#servings/day). NEFA 14:0 and notably 15:0 are selective biomarkers of dairy fat since they were not correlated to either low-fat or fermented dairy intake. Also, circulating NEFA 15:0 status did not differentiate dietary sub-groups of pregnant women from FAMILY (p = 0.36) as they had similar consumption patterns for full-fat dairy (**Table 1**) with a median serum concentration of 2.78 μM for 15:0 as its NEFA.

Dietary Intervention Study in Women: FO Supplementation and Serum NEFA Trajectories

In this study, serum NEFAs were analyzed in fasting serum samples from 18 young women collected at 4 time points over a 56 day intervention period, including baseline. For the active treatment arm, there was a mean 2.5-fold increase in serum NEFA concentrations for [EPA+DHA] from baseline after 28 days following high-dose FO supplementation as compared to the placebo group; however, there were no further changes in serum concentrations of EPA, DHA, or [EPA+DHA] at later sampling times (42 and 56 days) when using a 2-way mixed model ANOVA (p = 0.012) as shown in **Figure 3A**. As expected, temporal concentrations did not change for other serum NEFA measured by MSI-NACE-MS either within-subjects, or between treatment arms at all time points (**Table 4**). This includes circulating linoleic acid and oleic acid despite being major constituents of sunflower oil consumed in the placebo group (**Figure 3B**); these two major FA in circulation are highly abundant in numerous other food sources in the diet. Overall, changes in serum EPA as its NEFA was found to be more sensitive to FO supplementation as compared to DHA or [EPA+DHA], which also was able to readily detect a non-adherent participant (S10) to FO supplementation previously reported not to be compliant with leg immobilization protocols (28). These outcomes are likely a result of the lower concentrations of EPA in circulation, and the higher dosage of EPA (≈ 3 g or 50% higher than DHA) used in FO supplement relative to DHA. Independent measurements available for FAs from erythrocyte membrane PL hydrolysate showed a mean fold-change in [EPA+DHA] concentration of 2.6 from baseline that was consistent with serum NEFA measurements. Further exploration of the underlying relationship between these two distinctive blood lipid pools revealed a strong correlation only for EPA (r = 0.66; p = 4.6 ×

10^{-10}) at all time points (n = 69) when comparing concentrations from NEFA (protein-bound) and erythrocytes (membrane-bound) PL fractions in matching blood samples unlike DHA (r = 0.22; p = 0.074) as depicted in **Figure 3C**. A moderate correlation for DHA was only evident when comparing baseline and control cases (r = 0.35; p = 0.015; n = 44) after excluding data from the ω-3 PUFA treatment arm post-supplementation. No associations were found for other FAs analyzed from these two blood fractions when using validated MSI-NACE-MS and GC-FID methods.

DISCUSSION

For the first time, we report that fasting serum NEFAs have promising utility for the monitoring of dietary fat intake and FO supplementation in women. Various blood fractions have been used for FA determination in nutritional studies (35, 36) ranging from circulating lipid pools involved in transport (*e.g.,* NEFA, serum phospholipid fraction), cellular function (*e.g.,* erythrocyte or platelet membrane), to long-term storage (*e.g.,* adipose tissue triacylglycerides) (37); however, reports on serum NEFAs as biomarkers of dietary fat have been sparse likely due to technical challenges in limiting background lipid hydrolysis even under mild reaction conditions for preparation of fatty acid methyl esters prior to GC analysis (38). GC methods offer excellent selectivity for resolution of FA and some geometric/positional isomers, but require longer analysis times (> 20 min) even when using optimal column and elution conditions for comprehensive FA determination (39). Alternatively, rapid serum/fasting NEFA screening can be achieved by an enzymatic-based colorimetric assay yet this less selective method is prone to bias with discordant results as compared to LC-MS methods (40). Also, separation-free direct infusion-high resolution MS (41) or multiplexed chemical isotope labeling with LC-MS (42) offer greater sample throughput, but these approaches are better suited for analysis of total hydrolyzed FAs from serum/plasma after sample processing. In this work, fasting serum NEFAs were directly analyzed using a multiplexed separation platform based on MSI-NACE-MS (26), which offers a higher throughput method for assessment of complex dietary patterns associated with a health-promoting Prudent diet (9). Equivalent or

better correlations to self-reported intake of fish/seafood, full-fat dairy, as well as FO supplementation was achieved for certain serum NEFAs as compared to their corresponding total FAs from serum hydrolysates or erythrocyte membrane PL fractions. Indeed, there are conflicting reports on the exact relationship of fasting blood NEFAs and FAs from adipose tissue (19, 43, 44), where circulating NEFAs may serve as a virtual surrogate for tissue biopsies, and thus more accessible biomarkers of habitual diet that also respond to acute changes in the intake of seafood and dairy products (45).

Overall, our results from the cross-sectional study are consistent with a subset of participants from the EPIC study, where EPA and DHA had moderate correlations to self-reported fish intake from FFQ based on either total hydrolysates from plasma PL fraction (r = 0.33 and 0.29, respectively) or erythrocyte-membrane PL fraction (r = 0.29 and 0.40, respectively) (46). In our work, correlations for fasting serum EPA and DHA as their NEFAs ranged from 0.36 to 0.46 indicating that they provide an analogous assessment of habitual fat intake without lipid fractionation and hydrolysis as required for FA determination from serum phospholipids (36). For the high-dose ω-3 PUFA supplementation study, our results demonstrated a relatively fast equilibration time for serum [EPA+DHA] as their NEFAs as reflected by a mean 2.5 fold-change from baseline within 28 days, which is consistent with acute changes in circulating blood lipid pools in previous FO intervention studies (37, 47, 48); this was a selective treatment effect as no other changes were measured in other serum NEFAs from either active treatment or sunflower oil placebo arms. We hypothesize that this fast equilibration may be a consequence of the high ω-3 PUFA dosage regime used in this study (5 g/day) that is greater than the average intake of fish for Canadians (0.1-0.7 seafood meals/week) or used in commercial supplements (≈ 1 g/day) (22, 48). Furthermore, the strong correlation between erythrocyte membrane PL and serum EPA as its NEFA over the duration of the study, but not DHA supports that EPA is more responsive to changes in dietary patterns, which is a consistent finding in both our observational and intervention studies. This is also in agreement with reports on other plasma fractions, where EPA responds to high-dose FO supplementation and cessation within 1 week (49) despite appreciable retroconversion of EPA to DHA (50). In our work,

serum EPA concentrations (median = 1.6 μM, range from 1.0 to 6.3 μM) were lower (p = 0.00010) in pregnant women than DHA (median = 6.1 μM, range from 3.8 to 27 μM) as their NEFA, which was more striking as compared to differences in their total serum hydrolysate (p = 0.0010) concentrations (**Figure 1**). Also, EPA as its NEFA was much more sensitive to detect self-reported FO supplement use among 4 pregnant women, as well as acute changes in non-pregnant women following high-dose ω-3 PUFA supplementation as compared to DHA or [EPA+DHA] (**Figure 3A**; **Tables 2**, **4**) while also revealing suspected dietary non-adherence for a participant. Furthermore, serum EPA as its NEFA was strongly correlated (r = 0.66, $p = 4.6 \times 10^{-10}$) to independently measured erythrocyte PL membrane concentrations unlike DHA (**Figure 3C**). Consequently, we propose fasting serum EPA as its NEFA as a robust and sensitive dietary biomarker that correlates well to long-term/habitual fish intake, as well as acute changes following FO supplementation. This is important given expanding interests in high dose ω-3 PUFA (either EPA+DHA or EPA only) for prevention of muscle atrophy (28), reduction of asthma and persistent wheezing (51), promotion of lean mass and bone growth in childhood (52), as well as reducing atherosclerotic cardiovascular disease risk in patients with hypertriglyceridemia (53). However, ω-3 PUFAs can have quite distinctive lipid composition impacting their bioavailability while also varying up to 10-fold in natural abundance when comparing oily fish (*e.g.,* mackerel, salmon, sardines) to other commonly consumed lean fish (*e.g.,* haddock, canned tuna, cod) and other seafood sources (*e.g.,* algae, krill, prawns) (54). Interestingly, the poor diet quality sub-group of pregnant women had an estimated total ω-3 PUFA of only 71 mg/day from self-reported FFQ as compared to 217 mg/day for the healthy eating sub-group, both which are still below 300 mg/day DHA as recommended by the International Society for the Study of Fatty Acids and Lipids Working Group (48). This information is valuable for prenatal screening of ω-3 PUFA nutritional status and reliable monitoring of individual responses to dietary modifications or supplementation regimes for optimal maternal health.

Next, OCFAs are of special interest due to their role as promising food-specific biomarkers of full-fat dairy intake, which have also been reported to be inversely associated with type 2 diabetes risk

(55, 56). Observational and intervention studies have reported that 15:0 and 17:0 are dietary biomarkers reflecting milk fat intake as measured from adipose tissue TG, serum PL, serum CE, total serum lipids, as well as dried blood spots (55-59); however, there are sparse reports from the analysis of serum OCFAs as their NEFAs (60). In fact, serum 17:0 does not correlate with 15:0 since 17:0 can also be endogenously synthesized via α-oxidation, as well as generated via propionate via the action of gut microbiota on fermentable fiber (55, 56); for these reasons, 17:0 may serve as a putative biomarker of dietary fiber intake. Alternatively, adipose tissue 14:0 has been proposed as a biomarker for long-term intake of dairy fat (59). Our results confirmed that 14:0 (r = 0.30, p = 0.034), and especially 15:0 (r = 0.43, p = 0.0020) as their NEFAs were robust dietary biomarkers of full-fat dairy intake in pregnant women (**Figure 2D**; **Table 4**), but not skim/low fat or fermented milk. Our results for 17:0 was inconsistent when comparing NEFAs and total FA hydrolysates, with only the latter showing a weak association with self-reported intakes of full-fat dairy and total fiber, including soluble and insoluble fiber. Further studies that incorporate microbiome analyses are needed to better elucidate the utility of OCFAs as biomarkers of fiber intake since it is a major source of biological variance. Nevertheless, our work validates the use of fasting serum NEFAs as a convenient circulating lipid pool reflecting dietary intake of oily fish and full-fat dairy without invasive adipose tissue biopsies.

Strengths of our study include use of a rapid method based on MSI-NACE-MS for quantitative serum NEFA determination with stringent quality control that was applied to two independent cohorts of women involving a validated FFQ, and a placebo-controlled, high-dose ω-3 PUFA clinical trial. Serum NEFAs feature rarely in nutritional studies when relying on low throughput GC protocols that are susceptible to background lipid hydrolysis and oxidation artifacts during sample processing. This work also compared analyses between serum NEFAs and total FA hydrolysates for assessment of long-term/habitual fat intake during pregnancy, as well as acute/short-term changes with high-dose ω-3 PUFA supplementation in women. Our method offers a convenient alternative to erythrocyte membrane PL hydrolysate analyses especially in large-scale epidemiological studies given the availability of serum or

plasma in biorepositories. This study has some limitations, including the modest sample size of each cohort involving a single biological sex, and the lack of self-reported diet records in the ω-3 PUFA clinical trial. Also, since extreme diet scores from pregnant women were selected to maximize the effect size, this might have introduced a selection bias. Similarly, we aimed at studying associations of serum NEFA concentrations with self-reported FFQ rather assessing health outcomes of pregnant women. Long-term stability studies for protein-bound NEFAs are also needed to rule out potential bias due to prolonged storage of frozen serum samples collected from FAMILY (> 10 years). For the intervention study, sampling shorter time points (< 28 days) is also needed to better assess the minimum time frame required for ω-3 PUFA equilibration that is likely both dose and sex-dependent. In conclusion, our study introduces a rapid yet inexpensive approach for quantification of serum NEFA that avoids serum lipid fractionation, hydrolysis and/or pre-column chemical derivatization procedures. This approach largely provides equivalent and in some cases superior results as compared to total FA hydrolyzed from serum, as well as erythrocyte PL fraction notably in the case of assessment of circulating EPA and 15:0 as optimal NEFA biomarkers of habitual intake of fish/fish oil and full-fat dairy, respectively. MSI-NACE-MS is anticipated to facilitate large-scale blood-based testing of serum NEFA with greater sample throughput, lower costs, and improved quality control than standard GC protocols. This is needed for more accurate assessment of dietary fat intake for evidence-based nutritional policies that promote maternal health.

Data Availability Statement

All processed serum FA data and de-identified clinical/dietary information for participants is available as an excel file in the supporting information section (NEFA-Serum-JLR.xlsx). This data includes original and batch-corrected RPA for NEFAs measured by MSI-NACE-MS, as well as their serum concentrations from both observational and intervention studies, including matching total serum hydrolysate and independent erythrocyte PL fraction measurements, respectively.

The authors declare no conflict of interest.

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Table 1. Anthropometric and clinical characteristics of a cross-section of second-trimester pregnant women with contrasting dietary patterns from the FAMILY study.

Parameter	Poor diet group (*n*=25)	Good diet group (*n*=25)	*p*-value
Age (years)	31.3 ± 5.2	33.7 ± 5.7	0.14
Pre-pregnancy BMI (kg/m^2)*	29.3 ± 7.4	24.6 ± 5.3	0.011
Gestational weight gain (kg)	14.8 ± 6.9	14.1 ± 4.4	0.69
Total cholesterol (mmol/L)	6.0 ± 1.2	6.9 ± 1.0	0.015
LDL (mmol/L)	3.1 ± 1.0	3.5 ± 1.3	0.32
HDL (mmol/L)	1.8 ± 0.4	2.2 ± 0.5	0.006
Triglycerides* (mmol/L)	2.2 ± 0.8	2.3 ± 0.9	0.76
Fasting blood glucose (mmol/L)	4.4 ± 0.4	4.4 ± 0.5	0.69
HbA1c*	0.05 ± 0.01	0.05 ± 0.003	0.65
Diet quality index score* (range)	- 9.1 ± 3.0 (-16.5 to - 6.2)	12.0 ± 1.9 (10.8 to 17.4)	< 0.0001
Fish/seafood (#servings/day)*	0.1 ± 0.1	0.3 ± 0.2	< 0.0001
Total [EPA+ DHA] (g/day)*	0.07 ± 0.06	0.39 ± 0.50	< 0.0001
Total fiber (g/day)	18.3 ± 8.3	34.2 ± 7.6	< 0.0001
Full-fat dairy (#servings/day)	1.2 ± 1.1	1.6 ± 1.4	0.36

Presented data are mean and error as ± 1 s. Statistical comparisons assuming equal (t-test) or unequal variance (Welch's t test) or non-parametric Mann-Whitney test were performed as appropriate. Results were considered significant when $p < 0.05$. Full range of diet quality index score shown.*

Table 2. Spearman rank correlation coefficients between serum ω-3 PUFA concentrations measured as their NEFA or total (hydrolyzed) FA fraction as compared to the diet quality index score, fish/seafood daily servings, and total ω-3 PUFA intake in pregnant women (*n*=50) with contrasting diets from the FAMILY study.

	Diet quality index score[a]		**Fish/seafood (#servings/day)**		**Total ω-3 (g/day)[b]**	
Serum fatty acids	*r*	*p-value*	*r*	*p-value*	*r*	*p-value*
NEFA						
EPA (20:5*n*–3)	0.29	0.043*	0.45	0.0010**	0.46	0.0010**
DHA (22:6*n*–3)	0.38	0.0060**	0.36	0.011*	0.40	0.0040**
[EPA+DHA]	0.36	0.010**	0.40	0.0040**	0.43	0.0020**
Total FA						
EPA (20:5*n*–3)	0.37	0.0090**	0.47	0.0010**	0.50	0.00030**
DHA (22:6*n*–3)	0.28	0.052	0.29	0.045*	0.33	0.024*
[EPA+DHA]	0.40	0.004**	0.43	0.0020**	0.46	0.0010**

*Correlation is significant at the * 0.05 level (2-tailed) ** 0.01 level (2-tailed)**

[a] *An aggregate score reflecting differences in nutritional quality of foods consumed based on the sum of the daily number of servings of healthy/nutrient-rich foods (e.g., fermented dairy, fish/seafood, vegetables, fruits, whole grains, nuts/seeds) minus the daily number of servings of unhealthy/processed foods (e.g., processed meats, refined grains, fries, snacks/sweets).*

[b] *Daily average servings of [EPA + DHA] intake estimated from FFQ, including diet (fish/seafood) and FO supplement use.*

Table 3. Spearman rank correlation coefficients between serum FA measured as their NEFA or total (hydrolyzed) FA fraction as compared to full-fat dairy daily servings, and total fiber in pregnant women (n = 50) with contrasting diets from the FAMILY study.

	Full-fat Dairy (#servings/day)[a]		**Total Fiber (g/day)[b]**	
Serum Fatty Acids	*r*	*p-value*	*r*	*p-value*
NEFA				
Myristic acid (14:0)	0.30*	0.034	0.040	0.76
Pentadecanoic acid (15:0)	0.43**	0.0020	0.12	0.39
Heptadecanoic acid (17:0)	0.21	0.15	-0.26	0.86
Total				
Myristic acid (14:0)	0.35*	0.016	0.11	0.48
Pentadecanoic acid (15:0)	0.33*	0.023	0.22	0.14
Heptadecanoic acid (17:0)	0.29*	0.043	0.29*	0.050

*Correlation is significant at the * 0.05 level (2-tailed) ** 0.01 level (2-tailed)*

[a] *Daily average servings of full-fat dairy products estimated from FFQ, including intake of cream of any kind, whole milk, milk, cottage/ricotta cheese, cream cheese, sour/whipping cream, full-fat cheese .*

[b] *Daily average intake of total fiber from various dietary sources estimated from FFQ.*

Table 4. Fasting serum NEFA identified using a 2-way mixed-model ANOVA with repeated time points for within-subjects effects between high dose FO supplementation and control (sunflower oil) groups.

	Test of within-subjects effects				**Test of between-subjects effects**			
Serum Fatty Acids	*F*	*p-value*[a]	*Effect size*[b]	*Study power*	*F*	*p-value*[a]	*Effect size*[b]	*Study power*
EPA	8.36	0.0020*	0.39	0.99	22.2	0.0004*	0.63	0.99
[EPA+DHA]	5.59	0.0030*	0.30	0.92	19.0	0.0010*	0.59	0.98
DHA	3.54	0.023*	0.21	0.74	13.1	0.0030*	0.50	0.92
Myristic acid	1.93	0.14	0.13	0.46	1.5	0.24	0.10	0.21
Pentadecanoic acid	1.28	0.30	0.090	0.31	0.0	0.95	0.00	0.05
Heptadecanoic acid	0.28	0.84	0.020	0.10	0.26	0.62	0.02	0.080
Linoleic acid	0.34	0.80	0.030	0.11	1.5	0.24	0.11	0.21
Oleic acid	0.50	0.68	0.040	0.13	1.9	0.19	0.13	0.25

[a] *Mixed ANOVA model significant at the 0.001 level for EPA and DHA only for within and between subjects effects, where data sphericity was assumed/satisfied using Mauchly's test of sphericity.*
[b] *Based on partial Eta squared*

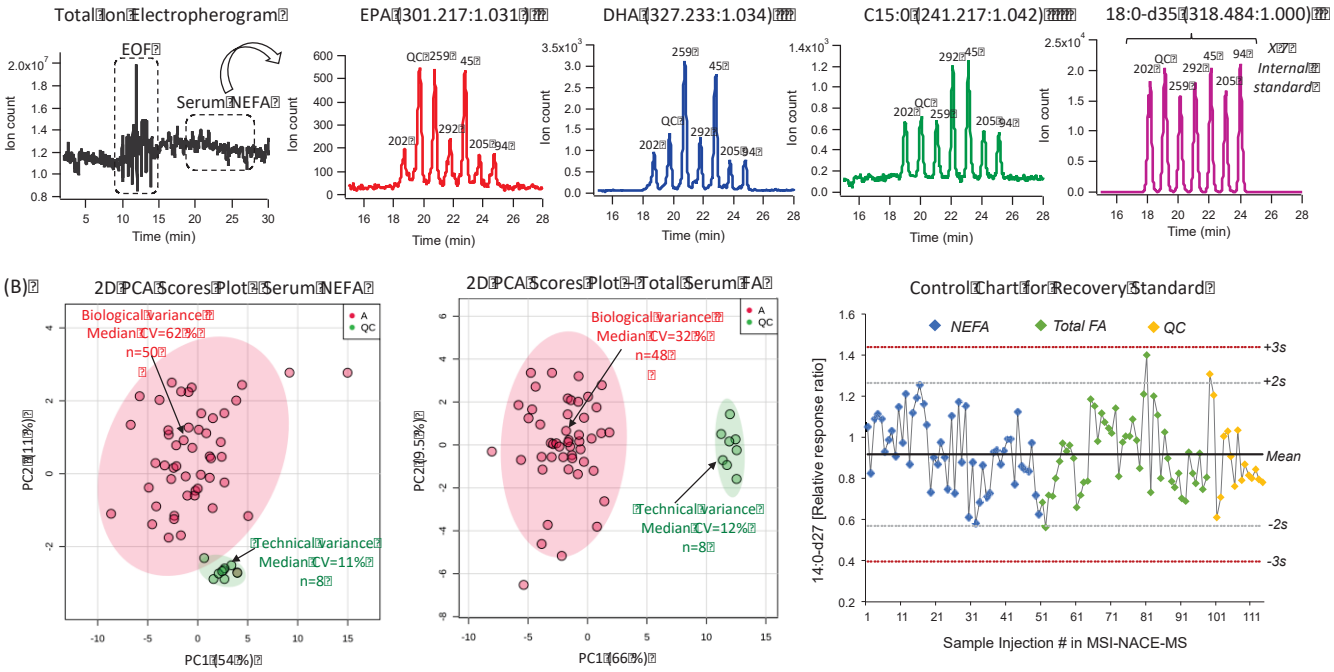

Fig. 1. (A) Multiplexed separations of FA from serum extracts for assessment of dietary fat intake in pregnant women using MSI-NACE-MS under negative ion mode detection. This method relies on a serial injection of six randomized samples and a QC within each run to enhance sample throughput, where traces depict a total electropherogram, and a series of extracted ion electropherograms for representative serum NEFA annotated by their characteristic accurate mass and relative migration time (*m/z*:RMT), including a deuterated internal standard for data normalization. (B) Unsupervised multivariate data analysis using PCA depicts the biological variance from 24 FA as their NEFA or total hydrolysates, as compared to the technical variance from repeat QC, including a control chart for a recovery standard added to all processed serum samples.

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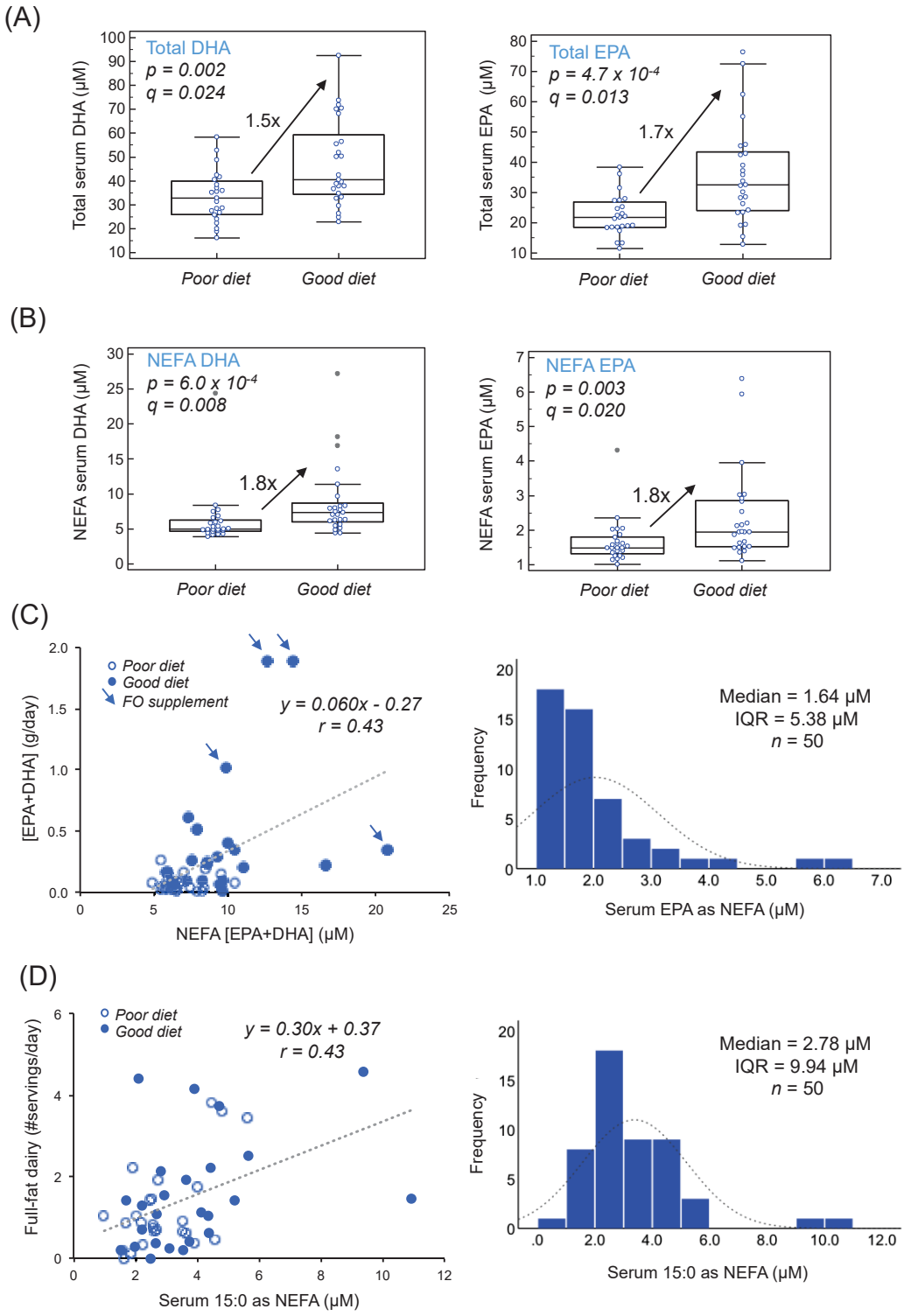

Fig. 2. Boxplots and scatter plots for ω-3 PUFA in serum as (A) total hydrolyzed FA and (B) NEFA that are different among pregnant women with contrasting diets based on a DQI score using univariate Mann-Whitney test ($p < 0.05$) and after FDR adjustments ($q < 0.05$). (C) Scatter plot (left) showing the correlation of serum [EPA+DHA] measured as their NEFA by MSI-NACE-MS with self-reported total daily intake of ω-3 PUFA from FFQ; and histogram (right) showing the concentration distribution of EPA as its NEFA. (D) Scatter plot (left) showing the correlation of 15:0 as its NEFA with daily servings of full-fat dairy from FFQ, and histogram (right) showing the concentration distribution of 15:0 as its NEFA.

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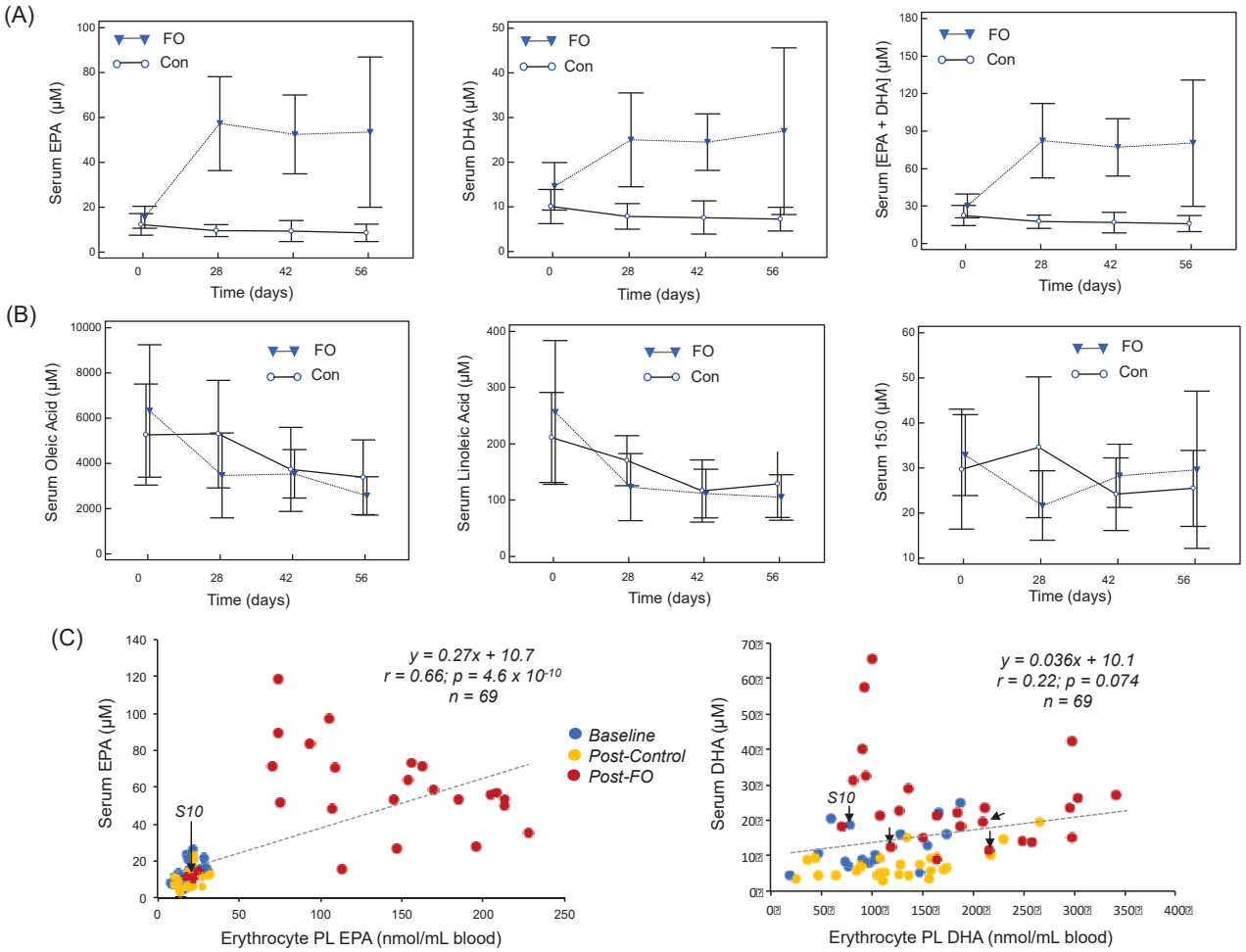

Fig. 3. Graphs depicting dynamic changes in serum NEFA concentrations in the intervention study in young women for the high dose fish oil supplementation group (FO) (n = 9) as compared to sunflower oil (Con) (n = 9) based on means and error bars (± 2s), including (A) responsive EPA, DHA, and [EPA+DHA] in contrast to (B) non-responsive serum NEFA controls, including oleic acid, linoleic acid and pentadecanoic acid. The former two FA are major constituents in sunflower oil used that did not change in the control group. (C) Scatter plots showing a strong linear correlation (r = 0.66) between EPA concentrations as its serum NEFA as compared to corresponding erythrocyte phospholipid (PL) fraction at all time points (n = 69), unlike the much weaker correlation (r = 0.22) for DHA concentrations. Serum EPA was also more sensitive to detect dietary non-adherence to FO supplementation as indicated by arrows for one participant (S10) as compared to DHA as its NEFA.

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