Compound-specific isotope analysis reveals no retroconversion of DHA to EPA but substantial conversion of EPA to DHA following supplementation: a randomized control trial

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ABSTRACT

Background: It has long been believed that DHA supplementation increases plasma EPA via the retroconversion pathway in mammals. However, in rodents this increase in EPA is likely due to a slower metabolism of EPA, but this has never been tested directly in humans.

Objective: The aim of this study was to use the natural variations in ^{13}C:^{12}C ratio (carbon-13 isotopic abundance [$\delta^{13}C$]) of n–3 PUFA supplements to assess n–3 PUFA metabolism following DHA or EPA supplementation in humans.

Methods: Participants (aged 21.6 ± 2.2 y) were randomly assigned into 1 of 3 supplement groups for 12 wk: *1*) olive oil control, *2*) ∼3 g/d DHA, or *3*) ∼3 g/d EPA. Blood was collected before and after the supplementation period, and concentrations and $\delta^{13}C$ of plasma n–3 PUFA were determined.

Results: DHA supplementation increased ($P < 0.05$) plasma EPA concentrations by 130% but did not affect plasma $\delta^{13}C$-EPA (−31.0 ± 0.30 to −30.8 ± 0.19, milliUrey ± SEM, $P > 0.05$). In addition, EPA supplementation did not change plasma DHA concentrations ($P > 0.05$) but did increase plasma $\delta^{13}C$-DHA (−27.9 ± 0.2 to −25.6 ± 0.1, $P < 0.05$) toward $\delta^{13}C$-EPA of the supplement (−23.5 ± 0.22). EPA supplementation increased plasma concentrations of EPA and docosapentaenoic acid (DPAn–3) by 880% and 200%, respectively, and increased plasma $\delta^{13}C$-EPA (−31.5 ± 0.2 to −25.7 ± 0.2) and $\delta^{13}C$-DPAn–3 (−28.9 ± 0.3 to −25.0 ± 0.1) toward $\delta^{13}C$-EPA of the supplement.

Conclusions: In this study, we show that the increase in plasma EPA following DHA supplementation in humans does not occur via retroconversion, but instead from a slowed metabolism and/or accumulation of plasma EPA. Furthermore, substantial amounts of supplemental EPA can be converted into DHA. $\delta^{13}C$ of n–3 PUFA in humans is a powerful and underutilized tool that can track dietary n–3 PUFA and elucidate complex metabolic questions. This trial was registered at clinicaltrials.gov as NCT03378232. *Am J Clin Nutr* 2019;00:1–9.

Keywords: docosahexaenoic acid, eicosapentaenoic acid, carbon-13 isotopic abundance, polyunsaturated fatty acid, metabolism, humans, isotope ratio mass spectrometry

Introduction

The n–3 PUFA biosynthesis pathway converts α-linolenic acid (ALA, 18:3n–3) to DHA via multiple elongation, desaturation, and β-oxidation steps, and includes the production of numerous intermediary PUFA. Although believed to be a reasonably well-understood pathway, our laboratory has recently made novel in-vivo discoveries in rats pertaining to the pathway including significant elongation of DHA to tetracosahexaenoic acid (24:6n–3) (1), and higher rates of n–3 PUFA synthesis with short-term DHA feeding (2). However, despite recent application of these in-vivo models in two human subjects (3), these studies require intravenous infusion of substantial amounts of expensive tracers that can make application to human populations difficult. Therefore, simpler methods for assessing the origin and metabolic fate of PUFA in humans are necessary. Our laboratory has recently utilized the natural variations in the ^{13}C:^{12}C ratio of the food supply (reported as carbon-13 isotopic abundance [$\delta^{13}C$]) to answer complex questions relating to the n–3 PUFA biosynthesis pathway (4–6). A more detailed description of the reported values is provided in the methods.

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The data used in the manuscript will be made available to editors upon request either before or after publication for checking.

Supplemental Figure 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AA, arachidonic acid; ALA, α-linolenic acid; $\delta^{13}C$, carbon-13 isotopic abundance; DPAn–3, n–3 docosapentaenoic acid; DPAn–6, n–6 docosapentaenoic acid; GC-FID, GC-flame ionization detection; GC-IRMS, GC-isotope ratio mass spectrometry; HUFA, highly unsaturated fatty acid that has ≥20 carbons and ≥3 double bonds; LA, linoleic acid: mUr, milliUrey.

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Plants are termed C_3 and C_4 due to differences in photosynthetic processes and resultant carbon fixation, and one outcome of these differences results in fatty acids (and other molecules) isolated from C_4 plants having naturally higher ^{13}C concentrations (−10 to −16 milliUrey [mUr]) compared with fatty acids isolated from C_3 plants (−23 to −32 mUr) (7). Furthermore, the carbons derived from aquatic organisms tend to yield more intermediate concentrations of ^{13}C in fatty acids (−16 to −23 mUr) (8). These natural variations in the $\delta^{13}C$ signature of fatty acids in the food supply have been detected in blood and tissues following the consumption of corn oil with a high $\delta^{13}C$ content and safflower oil with a low $\delta^{13}C$ content in mice (4), and following the consumption of DHA with an intermediate $\delta^{13}C$ content and ALA with a low $\delta^{13}C$ content in mice (9) and rats (5). In addition, feeding corn oil with a high $\delta^{13}C$ content to infants has been used to estimate the percentage conversion of linoleic acid (LA, 18:2n–6) to arachidonic acid (AA, 20:4n–6) (10), and high $\delta^{13}C$-DHA and $\delta^{13}C$-AA in formula fed to infants has been used to estimate synthesis rates of plasma phospholipid AA and DHA (11). These studies elucidate the wide-ranging potential benefits for the utilization of natural $\delta^{13}C$ differences in the food supply for the assessment of numerous biological, metabolic, and dietary assessment questions in humans. Not only has $\delta^{13}C$ recently been identified as a valuable biomarker for fish/seafood and animal protein intake (12), but a wider call for the expanded use of these biomarkers to estimate intake of foods or dietary patterns has been made (13). The present study is a secondary analysis from a double-blind, randomized control trial where the primary endpoint assessing the effectiveness of EPA or DHA supplements on the omega-3 index (percentage EPA + DHA in erythrocytes) was reported (14). Our study is the first to utilize the natural variations in $\delta^{13}C$ of EPA and DHA supplements to assess the metabolism of the n–3 PUFA biosynthetic pathway following consumption of EPA or DHA supplements in humans.

TABLE 1 Fatty acid composition and $\delta^{13}C$ of oils contained in supplements[1]

	Supplement		
Fatty acid	Olive oil	EPA	DHA
16:0	11.1 ± 0.02	0.23 ± 0.02	2.79 ± 0.09
18:0	2.98 ± 0.01	2.38 ± 0.03	0.93 ± 0.03
20:0	0.37 ± 0.001	0.46 ± 0.01	0.09 ± 0.01
SFAs	14.5 ± 0.02	3.08 ± 0.05	3.82 ± 0.12
16:1n–7	0.76 ± 0.002	—	0.2 ± 0.02
18:1n–7	1.73 ± 0.01	0.94 ± 0.003	0.45 ± 0.07
18:1n–9	75.7 ± 0.01	1.61 ± 0.01	15.7 ± 2.55
δ^{13}C-18:1n–9	*−29.0 ± 0.28*	*−26.5 ± 0.15*	*−29.7 ± 0.11*
20:1n–9	0.25 ± 0.0005	3.08 ± 0.02	0.08 ± 0.01
22:1n–9	—	0.24 ± 0	—
MUFAs	78.4 ± 0.01	5.86 ± 0.03	16.4 ± 2.6
18:2n–6	5.74 ± 0.001	0.47 ± 0.01	1.23 ± 0.13
δ^{13}C-18:2n–6	*−30.6 ± 0.19*	—	—
20:2n–6	0.11 ± 0.003	0.34 ± 0	—
20:3n–6	—	0.65 ± 0.01	—
20:4n–6	—	4.84 ± 0.01	—
δ^{13}C-20:4n–6	—	*−22.0 ± 0.19*	—
22:5n–6	—	—	0.76 ± 0.01
n–6 PUFA	5.85 ± 0.003	6.3 ± 0.01	1.99 ± 0.12
18:3n–3	0.65 ± 0.0004	0.89 ± 0	0.15 ± 0.01
δ^{13}C-18:3n–3	*−31.5 ± 0.16*	—	—
20:5n–3	—	74.7 ± 0.09	1.05 ± 0.11
δ^{13}C-20:5n–3	—	*-23.5 ± 0.22*	*−26.0 ± 0.80*
22:5n–3	—	—	1.46 ± 0.02
δ^{13}C-22:5n–3	—	—	*−25.6 ± 0.41*
22:6n–3	—	0.55 ± 0.01	72.3 ± 1.28
δ^{13}C-22:6–3	—	*−23.6 ± 0.24*	*−27.5 ± 0.03*
n–3 PUFA	0.65 ± 0.0004	76.1 ± 0.08	75 ± 1.35

[1] Values are means ± SEM, $N = 3$. Fatty acid composition expressed as the percentage of fatty acid in total fatty acids. Fatty acid $\delta^{13}C$ are identified in italics and expressed as milliUrey. $\delta^{13}C$, Carbon-13 isotopic abundance

Methods

Participants and study design

The study participants (45 females, 45 males, aged 21.6 ± 2.2 y, BMI 23.6 ± 3.2 kg/m^2) were recruited from the University of Guelph (Guelph, Ontario, Canada). Eligible participants were required to be aged between 18 and 30 y and healthy. Exclusion criteria included: age, pregnancy, high n–3 consumption, chronic or communicable diseases, use of chronic anti-inflammatory medication, use of lipid-controlling medications, allergies to fish, shellfish, or gelatin, anticipated changes in lifestyle, or discomfort giving blood. Participants were randomly assigned to groups by a computer-generated random number list, using block randomization with stratification by sex (14). Through sequential numbering of plasma storage vials, all participants, researchers, phlebotomists, and laboratory personnel were blinded throughout the intervention period and until after data generation. The study was registered at clinicaltrials.gov (NCT03378232) with primary (omega-3 index) and secondary (heart rate, blood pressure, and muscle sympathetic nerve activity) endpoints, and sample size calculations previously described (14).

All participants provided written, informed consent and the study was approved by the Human Research Ethics Board at the University of Guelph. In a double-blinded, parallel group design, participants were randomly assigned to 1 of 3 equally distributed supplementation groups: *1*) an olive oil control ($n = 30$), *2*) an EPA supplementation ($n = 30$), or *3*) a DHA supplementation ($n = 30$) group and were instructed to consume 4 capsules per day for a total of 12 wk. Following an overnight fast, participants visited the Human Nutraceutical Research Unit at the University of Guelph for blood sampling before and after the 12-wk supplementation period. Additional study details are provided in a Consolidated Standards of Reporting Trials flow diagram (**Supplemental Figure 1**).

Supplements

Olive oil for control supplements and purified EPA and DHA oils were obtained from KD Pharma. For blinding purposes, encapsulation was performed by InnovaGel for all supplements to be visually identical. Fatty acid compositions and $\delta^{13}C$ of selected fatty acids of each oil were determined and are reported in **Table 1**. Fatty acid purity was determined to be 75.7 ± 0.01% for 18:1n–9 in the olive oil control group, 74.7 ± 0.09% EPA in the EPA group, and 72.3 ± 1.3% DHA in the DHA group. However, we determined that a small amount of DHA (0.55 ± 0.01%) was present in the EPA supplement, and EPA (1.05 ± 0.11%) was present in the DHA supplement. These compositions represent intake concentrations of ∼3 g/d of EPA or DHA for each n–3

PUFA supplementation group and are generally considered safe at these concentrations.

Blood collection

Blood was collected from the antecubital vein into EDTA-treated vacutainers (LifeLabs). Blood fractions were separated by centrifugation at 3,000 × *g* for 15 min, and ∼500 μL of the upper plasma layer was aliquoted and stored at −80°C for 6–10 mo prior to analysis. Plasma samples were shipped to the University of Toronto on dry ice and stored at −80°C until lipid extraction.

Lipid extraction and transesterification

Lipids were extracted from 300 μL of plasma using a modified Folch method (15), as described previously by our laboratory (16). Briefly, lipids were extracted with 6 mL of 2:1 chloroform:methanol containing 10 μg of docosatriaenoic acid ethyl ester (22:3n–3, Nu-Chek Prep, Inc.) as an internal standard. Extracted lipids containing an internal standard were then transesterified to FAMEs with 14% boron trifluoride in methanol (17), as described previously. FAMEs in hexane were dried down, reconstituted in heptane, and analyzed by GC-flame ionization detection (GC-FID).

GC-FID

FAMEs were analyzed on a Varian 430 gas chromatograph (Scion Instruments) equipped with an SP-2560 biscyanopropyl siloxane, capillary column (100 m length × 0.25 mm diameter × 0.20 μm film thickness; Supelco), as previously described (9, 18). Peaks were identified using retention times by comparison to an external FAME reference standard mixture containing an equal weighting of FAME standards (GLC-569, Nu-Chek Prep, Inc.), and FAME responses were determined to be equivalent within experimental error and no response factors were applied. The concentration of each fatty acid was determined in relation to the known amount of 22:3n–3 ethyl ester internal standard present in the samples. After the samples were injected into the GC-FID, vials were recapped and stored at −80°C until analysis by GC-isotope ratio mass spectrometry (GC-IRMS).

Isotopic analysis

The plasma δ^{13}C of the FAMEs were determined by GC-IRMS, as described previously (4, 5, 9, 18). For optimal separation of DHA peaks from cholesterol, an alternate temperature program compared with the GC-FID program was necessary. Complete separation was achieved with the following program: initial temperature of 60°C with an immediate ramp of 8.5°C/min to 170°C with a 6.1-min hold, a 4.3°C/min ramp to 175°C with no hold, a 1.7°C/min ramp to 185°C with no hold, a 0.8°C/min ramp to 190°C with no hold, and finally, a 8.5°C/min ramp to 240°C with a 35-min hold. The column flow rate was set to 1.0 mL/min.

Reporting of carbon-13 isotopic values

The values universally used for the reporting of the ^{13}C content of natural samples including biological tissues have been considered nonintuitive since being first proposed (19). Generally, ^{13}C isotopic values are reported as the difference in the ^{13}C:^{12}C ratio of a specific metabolite compared with the ^{13}C:^{12}C ratio of a universal reference material, identified as δ^{13}C and reported in milliUrey (mUr) (20). Each 1 mUr change is representative of a one per mille (1 in 1,000, ‰) change in the ^{13}C:^{12}C ratio compared with the reference material. Values are referenced to the carbonaceous remains of a Cretaceous marine fossil, *Belemnitella americana* (21), containing a high δ^{13}C value that is characteristic of carbonates. As a result, all living things, including nutrients derived from them, have negative δ^{13}C as they have a lower ^{13}C content when compared with *Belemnitella americana* (7). The δ^{13}C of plasma n–3 PUFA in our study will then have a higher ^{13}C content if the value is less negative (∼ −24 mUr), and a lower ^{13}C content if the value is more negative (∼ −30 mUr).

Isotopic normalization

δ^{13}C of plasma PUFA collected by GC-IRMS was normalized and converted to the international carbon isotope reference scale, Vienna Peedee Belemnite, by multipoint linear normalization and reported in mUr (4, 5, 9, 18). Certified calibrated 20-carbon FAME reference materials USGS70, USGS71, and USGS72 (Reston Stable Isotope Laboratory, United States Geological Survey) were injected at least once each during the sequences. Linear regression of measured values compared with true values (−30.53 ± 0.04, −10.50 ± 0.03, and −1.54 ± 0.03 mUr for USGS70, USGS71, and USGS72, respectively) was used to generate the normalizing equation to report δ^{13}C values for all data. R^2 values for all normalizing equations were >0.9998.

Methyl correction

Isotopic analysis by GC-IRMS provides data on carbon dioxide (CO_2) produced from the combustion of individual FAMEs, and therefore measurements include the isotopic contribution of carbon from the derivatized methyl group. To account for the contribution of the derivatized carbon, a methyl correction calculation was performed as previously described in detail (5, 9). For the correction, we compared isotopic signatures of nonadecanoic acid (19:0, NuChek Prep) and nonadecanoate methyl esters prepared using the same stock solution of 14% boron trifluoride in methanol that was used for sample transesterification.

Statistics

All statistical analyses were performed with IBM SPSS Statistics 24 software. Differences between pre- and postsupplementation fatty acid concentrations and carbon isotope ratios were determined by paired *t*-tests, and Pearson correlations were determined for the changes measured in carbon isotope signatures between fatty acids. Data that were not normally distributed as determined by the Shapiro–Wilk test were log-transformed prior to statistical analysis. Significance for all statistical analysis was determined at $P < 0.05$. All data are presented as means ± SEM.

Results

Participants

Ninety individuals participated in the study between January and June 2017, with 89 completing the full study protocol. One participant from the EPA supplementation group was unavailable for the 12-wk blood sampling and was removed from the study analysis. Final analyses for plasma fatty acid concentrations and δ^{13}C included 30 in the olive oil control group, 29 in the EPA supplement group, and 30 in the DHA supplement group. No participants reported any adverse effects of the intervention. Baseline participant characteristics for each group, including sex, age (y), height (cm), weight (kg), BMI (kg/m^2), systolic and diastolic BP (mmHg), and heart rate (bpm) have been reported previously (14).

Plasma fatty acid concentrations following supplementation

The effect of 12 wk of olive oil (control), EPA, or DHA supplementation on plasma fatty acid concentrations were determined and are reported (**Table 2**). Firstly, olive oil supplementation had no effect ($P > 0.05$) on plasma concentrations of any n–3 or n–6 PUFA, total SFA, total MUFA, or total fatty acids. However, relatively small increases ($P < 0.05$) in 20:0, 22:0, 24:0, and 24:1n–9 were detected.

EPA supplementation yielded significant increases ($P < 0.05$) in plasma EPA and DPAn–3 concentrations of 880% and 200%, respectively. There was no effect ($P > 0.05$) of EPA supplementation on plasma DHA concentrations. Total plasma n–3 PUFA was 150% higher ($P < 0.05$) following the supplementation period. Relatively small but significant decreases ($P < 0.05$) in plasma LA, dihomo-γ-linolenic acid (DGLA, 20:3n–6), n–6 docosapentaenoic acid (22:5n–6, DPAn–6), and total n–6 PUFA were also demonstrated following EPA supplementation. However, EPA supplementation had no effect ($P > 0.05$) on plasma total SFAs, total MUFAs, or total fatty acids.

DHA supplementation yielded 130%, 180%, and 100% higher ($P < 0.05$) plasma EPA, DHA, and total n–3 PUFA, as well as decreases ($P < 0.05$) in plasma 20:3n–3 and DPAn–3. Additional decreases ($P < 0.05$) in plasma γ-linolenic acid (GLA, 18:3n–6), 20:2n–6, 20:3n–6, AA, 22:4n–6, DPAn–6, and total n–6 PUFA were determined following DHA supplementation. Furthermore, DHA supplementation significantly decreased plasma 14:0, 16:0, 16:1n–7, 18:1n–7, 18:1n–9, 20:1n–9, and total MUFA, and increased ($P < 0.05$) 20:0, 22:0, 24:0, and 24:1n–9. Total fatty acids were lower ($P < 0.05$) after DHA supplementation; however, when considering pool size by calculating weight percentage of fatty acids in total fatty acids, only 14:0 and 22:4n–6 lost significance and 18:0 gained significance compared with concentrations (data not shown).

Changes in δ^{13}C of ALA, EPA, DPAn–3, and DHA following supplementation

The effect of olive oil, EPA, and DHA on the δ^{13}C of plasma ALA, EPA, DPAn–3, and DHA (**Figure 1**) were determined pre- and postsupplementation. These figures also indicate the dietary δ^{13}C of 18:1n–9, EPA, and DHA as a point of reference when presenting the changes of plasma δ^{13}C-n–3 PUFA, as reported in Table 1. Olive oil supplementation had no effect ($P > 0.05$) on the δ^{13}C of any n–3 PUFA measured. EPA supplementation significantly increased ($P < 0.05$) the δ^{13}C of plasma EPA from -31.5 ± 0.2 (mUr ± SEM) to -25.7 ± 0.2, of DPAn–3 from -28.9 ± 0.3 to -25.0 ± 0.1, and of DHA from -27.9 ± 0.2 to -25.6 ± 0.1. There was no effect ($P > 0.05$) of EPA supplementation on δ^{13}C of plasma ALA (-31.1 ± 0.2 to -30.7 ± 0.2). DHA supplementation increased ($P < 0.05$) δ^{13}C of plasma DPAn–3 from -28.4 ± 0.3 to -26.6 ± 0.4. No effects ($P > 0.05$) of DHA supplementation on the δ^{13}C of plasma ALA, EPA, or DHA were observed.

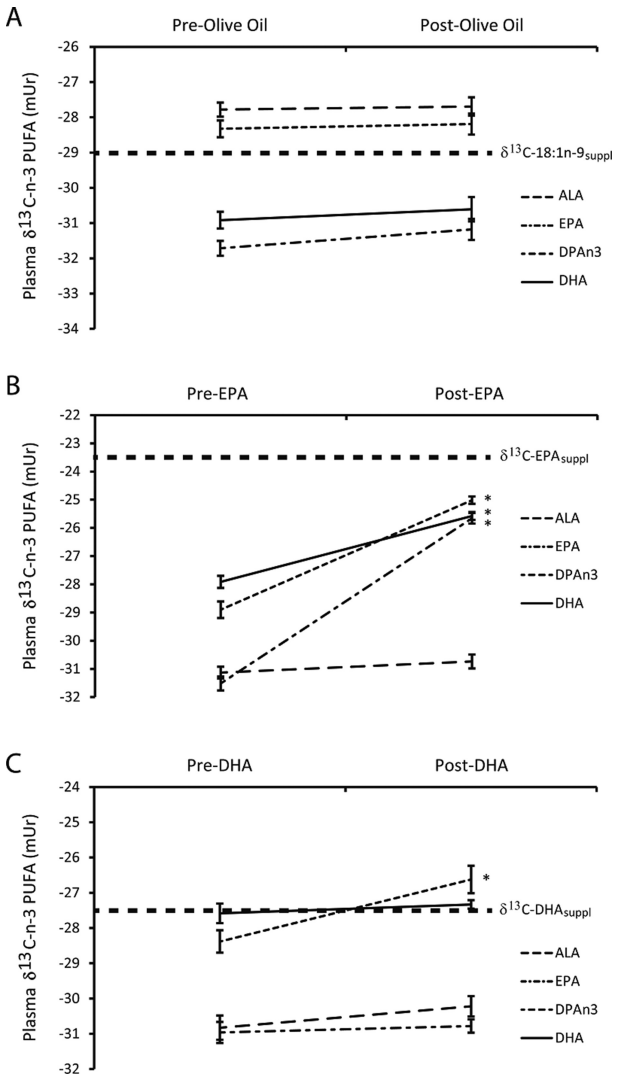

FIGURE 1 Plasma δ^{13}C-n–3 PUFA before and after (A) olive oil, (B) EPA, and (C) DHA supplementation for 12 wk; ∗represents significant effect of supplementation on plasma δ^{13}C-n–3 PUFA as determined by the paired *t*-test, $P < 0.05$. $N = 29$–30, means ± SEM. ALA, α-linolenic acid, 18:3n–3; δ^{13}C, carbon-13 isotopic abundance; DPAn–3, n-3 docosapentaenoic acid, 22:5n–3; mUr, milliUrey.

Correlation of changes in concentration compared with δ^{13}C of plasma 18:1n–9, EPA, and DHA

The correlation between changes in plasma concentrations and plasma δ^{13}C of the primary supplemented fatty acids 18:1n–9

TABLE 2 Plasma fatty acid concentrations before and after olive oil, EPA, or DHA supplementation for 12 wk

	Olive oil		EPA		DHA	
Fatty acid	Pre	Post	Pre	Post	Pre	Post
14:0	96.3 ± 10.6	110 ± 11	80.8 ± 7.1	103 ± 11	88.6 ± 8.8	73.2 ± 5.8*
16:0	2450 ± 135	2550 ± 121	2382 ± 105	2429 ± 98	2343 ± 78	2175 ± 75*
17:0	31.1 ± 1.7	32.7 ± 1.5	30.1 ± 1.1	31.7 ± 1.3	30.4 ± 1.0	29.0 ± 0.9
18:0	682 ± 30	733 ± 30	697 ± 25	731 ± 25	694 ± 22	709 ± 23
20:0	12.8 ± 0.5	15.5 ± 0.8*	13.9 ± 0.5	14.5 ± 0.9	13.8 ± 0.6	16.2 ± 0.8*
22:0	25.7 ± 1.3	31.2 ± 2.2*	28.3 ± 1.0	32.0 ± 2.3	27.6 ± 1.4	34.4 ± 2.1*
24:0	21.4 ± 1.2	25.7 ± 1.8*	23.1 ± 0.8	26.7 ± 1.9	22.8 ± 1.1	28.4 ± 1.7*
Total SFA	3359 ± 175	3540 ± 164	3289 ± 132	3409 ± 132	3258 ± 105	3097 ± 103
14:1n–5	5.57 ± 0.88	5.64 ± 0.86	4.47 ± 0.74	5.38 ± 0.76	4.86 ± 0.8	3.40 ± 0.41
16:1n–7	209 ± 24	193 ± 21	181 ± 16	178 ± 13	168 ± 11	119 ± 9*
18:1n–7	155 ± 8	155 ± 5	170 ± 19	145 ± 7	150 ± 7	121 ± 4*
18:1n–9	2087 ± 113	2204 ± 110	1972 ± 90	1896 ± 93	2064 ± 62	1766 ± 59*
20:1n–9	18.6 ± 1.2	21.1 ± 1.2	17.2 ± 0.8	16.4 ± 0.9	18.0 ± 0.9	14.6 ± 0.7*
22:1–9	3.02 ± 0.66	2.31 ± 0.19	2.15 ± 0.15	2.07 ± 0.13	3.21 ± 0.76	1.87 ± 0.10
24:1n–9	32.7 ± 1.2	38.4 ± 2.7*	35.9 ± 1.7	42.9 ± 3.1*	35.1 ± 1.9	41.6 ± 3.0*
Total MUFA	2511 ± 140	2538 ± 149	2382 ± 114	2286 ± 112	2442 ± 73	2067 ± 69*
18:2n–6	3304 ± 147	3484 ± 133	3219 ± 111	2942 ± 112*	3271 ± 99	3196 ± 103
18:3n–6	33.9 ± 2.9	36.2 ± 2.7	32.2 ± 2.4	29.0 ± 3.1	38.5 ± 4.5	23.1 ± 2.0*
20:2n–6	25.5 ± 1.4	29.7 ± 2.7	25.4 ± 3.2	21.5 ± 1.3	23.8 ± 0.9	19.6 ± 0.8*
20:3n–6	154 ± 11	164 ± 9	141 ± 9	104 ± 7*	141 ± 7	102 ± 6*
20:4n–6	739 ± 41	725 ± 37	723 ± 30	690 ± 21	716 ± 29	535 ± 24*
22:2n–6	1.44 ± 0.32	1.79 ± 0.36	2.34 ± 0.66	3.98 ± 0.20*	1.85 ± 0.34	1.91 ± 0.36
22:4n–6	34.6 ± 3.5	37.2 ± 2.4	35.9 ± 1.4	33.7 ± 2.7	36.3 ± 2.6	30.2 ± 2.5*
22:5n–6	22.7 ± 1.5	21.7 ± 1.7	19.9 ± 0.9	12.6 ± 0.8*	18.6 ± 1.0	12.0 ± 0.8*
Total n–6 PUFA	4315 ± 186	4500 ± 164	4199 ± 132	3836 ± 132*	4246 ± 125	3919 ± 122*
18:3n–3	75.5 ± 5.7	85.8 ± 5.8	71.3 ± 4.6	77.6 ± 4.5	76.7 ± 5.0	66.9 ± 3.8
20:3n–3	2.85 ± 0.25	3.28 ± 0.27	2.60 ± 0.18	2.91 ± 0.20	2.73 ± 0.17	2.24 ± 0.12*
20:5n–3	54.1 ± 5.7	60.0 ± 6.0	46.5 ± 3.2	454 ± 31*	58.8 ± 5.8	137 ± 10*
22:5n–3	37.3 ± 2.1	37.4 ± 2.1	39.6 ± 2.0	119 ± 7*	38.3 ± 2.1	24.6 ± 1.2*
22:6n–3	180 ± 12	188 ± 12	179 ± 9	183 ± 12	169 ± 9	473 ± 26*
Total n–3 PUFA	349 ± 22	374 ± 16	339 ± 14	836 ± 42*	345 ± 17	705 ± 33*
Total PUFA	4665 ± 204	4874 ± 174	4538 ± 142	4673 ± 144	4591 ± 135	4623 ± 143
Total HUFA	1224 ± 66	1237 ± 51	1188 ± 43	1599 ± 54*	1180 ± 43	1316 ± 52*
n–6/n–3 PUFA	12.9 ± 0.4	12.3 ± 0.4	12.7 ± 0.5	4.87 ± 0.27*	12.9 ± 0.6	5.82 ± 0.28*
% n–3 HUFA	22.4 ± 0.7	23.6 ± 0.8	22.5 ± 0.5	46.9 ± 1.3*	22.7 ± 0.8	48.2 ± 1.3*
Total FA	10,535 ± 500	10,952 ± 448	10,210 ± 366	10,367 ± 371	10,292 ± 294	9787 ± 302*

*Represents statistically significant effect of supplementation on plasma fatty acid concentration within olive oil, EPA, or DHA supplemented groups as determined by paired *t*-test, $P < 0.05$. $N = 29$–30, nmol/mL ± SEM. FA, fatty acid; HUFA, highly unsaturated fatty acid that has ≥20 carbons and ≥3 double bonds; % n–3 HUFA, percentage of n–3 highly unsaturated fatty acids (HUFA) in total HUFA.

(olive oil), EPA, and DHA were determined (**Figure 2**). The change in an individual's plasma EPA concentration was directly and positively correlated to the change in plasma δ^{13}C-EPA ($r = 0.56$, $P < 0.01$, Figure 2B) in the EPA-supplemented group. There were no significant correlations between plasma 18:1n–9 concentration and plasma δ^{13}C-18:1n–9 ($r = 0.25$, $P > 0.05$) in the olive oil– supplemented group (Figure 2A), or between plasma DHA concentrations and plasma δ^{13}C-DHA ($r = 0.22$, $P > 0.05$) in the DHA-supplemented group (Figure 2C).

Correlation of changes in δ^{13}C of plasma EPA and DHA with changes in δ^{13}C of plasma n–3 PUFA

The correlation between the changes in plasma δ^{13}C-EPA compared with changes in δ^{13}C of plasma ALA, DPAn-3, and DHA following EPA supplementation (**Figure 3**) and the correlation between the changes in plasma δ^{13}C-DHA compared with changes in δ^{13}C of plasma ALA, EPA, and DPAn–3 following DHA supplementation (**Figure 4**) are presented. Following EPA supplementation, the individual changes in plasma δ^{13}C-EPA for participants were positively correlated with changes in plasma δ^{13}C-DPAn–3 ($r = 0.46$, $P < 0.05$) and δ^{13}C-DHA ($r = 0.59$, $P < 0.001$), but not δ^{13}C-ALA ($r = 0.32$, $P = 0.097$) (Figure 3). Alternatively, following DHA supplementation, the changes in plasma δ^{13}C-DHA for individual participants were positively correlated only with changes in plasma δ^{13}C-DPAn–3 ($r = 0.48$, $P < 0.01$), and not plasma δ^{13}C-ALA ($r = -0.09$, $P = 0.65$) or δ^{13}C-EPA ($r = 0.12$, $P = 0.51$).

Discussion

In this study, individuals consumed olive oil, EPA, or DHA supplements for 12 wk. Plasma collected prior to and following the supplementation period was assessed for fatty acid concentrations and δ^{13}C of individual n–3 PUFA. The

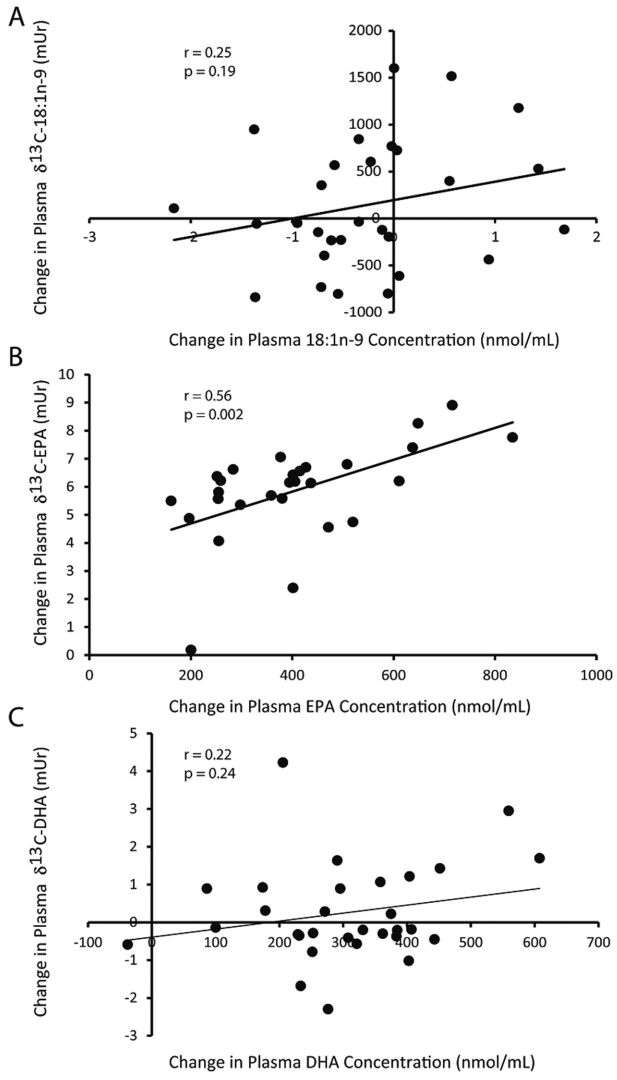

FIGURE 2 Correlation between the change in (A) plasma 18:1n–9 concentration compared with δ^{13}C-18:1n–9, (B) plasma EPA concentration compared with δ^{13}C-EPA, and (C) plasma DHA concentration compared with δ^{13}C-DHA before and after supplementation for 12 wk. Statistically significant correlations were determined by Pearson correlations, $P < 0.05$. $N = 29$–30, means ± SEM. δ^{13}C, carbon-13 isotopic abundance; mUr, milliUrey.

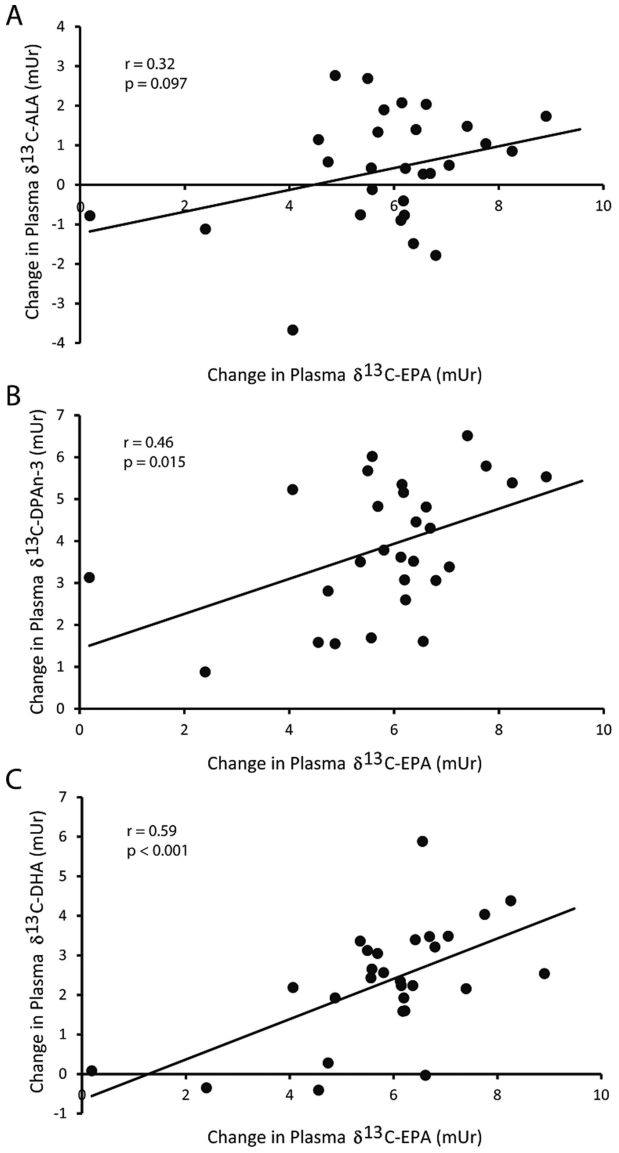

FIGURE 3 Correlation between the change in plasma δ^{13}C-EPA with the change in plasma (A) δ^{13}C-ALA, (B) δ^{13}C-DPAn–3, and (C) δ^{13}C-DHA before and after EPA supplementation for 12 wk. Statistically significant correlations were determined by Pearson correlations, $P < 0.05$. $N = 29$. ALA, α-linolenic acid, 18:3n–3; δ^{13}C, carbon-13 isotopic abundance; DPAn–3, n–3 docosapentaenoic acid, 22:5n–3; mUr, milliUrey.

δ^{13}C-DHA of the DHA supplement was not detected in plasma EPA and demonstrates for the first time in humans that slower EPA turnover, and not retroconversion, is the mechanism responsible for the concomitant rise in plasma EPA following DHA supplementation. Conversely, the δ^{13}C-EPA of the EPA supplement was detected in the plasma δ^{13}C-DHA, indicating substantial levels of in-vivo synthesis of DHA from EPA.

Consumption of DHA increases not only blood and tissue DHA but also EPA across a variety of species, including humans (16, 22–27). Retroconversion of DHA to EPA is thought to occur in rodents (28, 29) and humans (25, 28, 30), and has long been postulated as the mechanism responsible for the described increases in EPA with DHA feeding. However, another study referred to this as "apparent retroconversion" and noted the potential role of a slowed metabolism of EPA following DHA supplementation (31). Furthermore, recent evidence from our laboratory using δ^{13}C determination of liver EPA following 12 wk of DHA feeding in rats suggests that the increase in EPA is primarily the result of a longer half-life of EPA, and not increased conversion of DHA to EPA (5). Although the supplement δ^{13}C-DHA was not different from plasma δ^{13}C-DHA and somewhat limited our study, it was different from plasma δ^{13}C-EPA and allowed for the assessment of retroconversion in our population. Our current study in humans shows a 130% increase in plasma EPA concentrations with DHA supplementation, but with no effect on the δ^{13}C-EPA, thereby confirming our findings in rats. If DHA was contributing significantly to the increases in plasma EPA then plasma δ^{13}C-EPA would increase toward the concentrations of plasma δ^{13}C-DHA. However, with no change in δ^{13}C-EPA and with the value remaining nearly identical to plasma

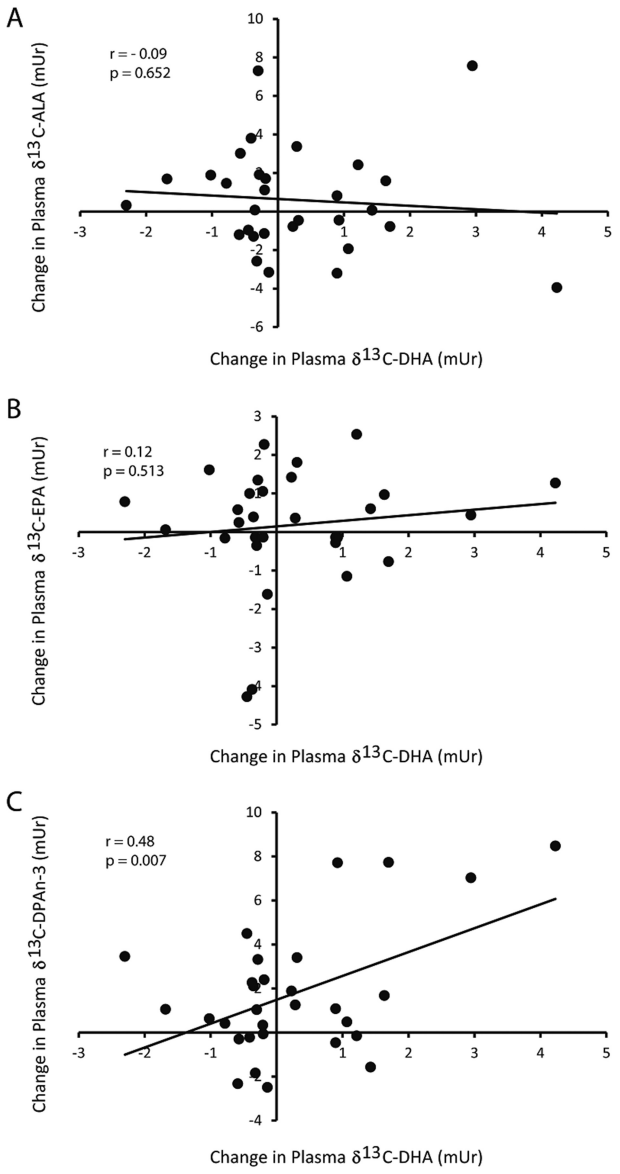

FIGURE 4 Correlation between the change in plasma δ^{13}C-DHA with the change in plasma (A) δ^{13}C-ALA, (B) δ^{13}C-EPA, and (C) δ^{13}C-DPAn–3 before and after DHA supplementation for 12 wk. Statistically significant correlations were determined by Pearson correlations, $P < 0.05$. $N = 30$. ALA, α-linolenic acid, 18:3n–3; δ^{13}C, carbon-13 isotopic abundance; DPAn–3, n–3 docosapentaenoic acid, 22:5n–3, mUr, milliUrey.

δ^{13}C-ALA, the source of the increased EPA concentrations is likely from ALA and the result of slower turnover/metabolism of plasma EPA. Our results in humans support our rodent studies and provide compelling evidence that retroconversion of DHA to EPA is not the primary mechanism responsible for the increases in EPA with DHA supplementation. Although it remains possible that there is a mobilization of EPA from tissue stores or displacement of EPA by DHA in membranes following DHA supplementation, rodent studies show very clearly that the increase in EPA occurs across numerous tissues as well as in plasma and erythrocytes (6, 24, 32).

Supplementation of EPA in humans continually results in no effect on plasma DHA concentrations (33–38), and is often considered as evidence for the very low or nonexistent metabolic conversion of EPA to DHA (39). Indeed, our participants showed no change in plasma DHA concentrations following 3 g/d EPA supplementation for 12 wk. However, the EPA provided in the supplement naturally contains a higher δ^{13}C-EPA (-23.5 ± 0.2 mUr) than that of the participant's plasma ALA, EPA, DPAn–3, and DHA. In response to the 12-wk supplementation period, the δ^{13}C of plasma EPA and the downstream metabolic products DPAn–3 and DHA increased significantly to values approaching that of δ^{13}C-EPA in the supplement. The δ^{13}C of plasma EPA, DPAn–3, and DHA are very similar, suggesting that following 12 wk of supplementation, the majority, if not all, of the plasma DPAn–3 and DHA was derived from the metabolic conversion of EPA. As a result, nearly all the endogenous plasma DHA that was present prior to EPA supplementation was replaced after 12 wk, and potentially sooner. However, one limitation of our study is that the EPA supplement contained 0.55% DHA, which was isotopically similar to the EPA in the supplement. Thus, although it would appear unlikely that such a low amount of DHA completely turned over the plasma DHA pool, we cannot rule out at least some contribution of carbons from the DHA in the EPA supplement to the plasma DHA pool.

The change in the plasma δ^{13}C-EPA from pre- to postsupplementation is highly correlated with the change for δ^{13}C-DHA and provides further evidence that substantially more EPA is metabolized to DHA than previously believed, but exactly how much is not yet clear. Combined with no change in plasma concentrations of DHA, our results provide evidence for the rapid turnover of DHA either through β-oxidation or conversion to downstream metabolic products (i.e., fatty acid ethanolamides or docosanoids) as the primary determinant of plasma DHA concentrations rather than synthesis. This is consistent with our previous finding from rodents infused with $^{2}H_5$-ALA and $^{13}C_{22}$-DHA (29). Future studies could test this hypothesis following an EPA supplementation period via the measurement of concentrations and δ^{13}C of blood DHA metabolites, such as docosanoids and ethanolamides. Furthermore, breath CO_2 following a single oral dose of isotopically labeled DHA following an EPA supplementation protocol could identify changes in β-oxidation of DHA (40). Future research could also determine the conversion of ALA to DHA using dietary δ^{13}C-ALA, as human conversion rates from dietary ALA are considered to be very low (41–50), and consuming as much as 15 g/d of ALA can have no effect on plasma DHA concentrations (39, 51).

Increases in plasma δ^{13}C-EPA to concentrations nearing that of δ^{13}C-EPA in the supplement of the current study support previous conclusions from our laboratory involving rodents that natural variations in δ^{13}C of the food supply can determine the dietary origins of blood and tissue n–3 PUFA (4, 5, 9). Adherence to advice to consume fish or fish oil can result in low compliance rates (52, 53), and improved methods for tracking adherence to advice are needed. The naturally higher δ^{13}C in marine-derived n–3 PUFA compared with terrestrial n–3 PUFA sources could be used to monitor adherence to dietary advice, with the change in plasma EPA concentrations of our study's participants directly correlated to the change in plasma δ^{13}C-EPA. However, the cost of and access to the necessary GC-IRMS equipment may currently be prohibitive for some researchers. Importantly, plasma δ^{13}C-DHA did not change pre- to postsupplementation

when consuming a DHA supplement with almost identical δ^{13}C-DHA (−27.5 ± 0.03 mUr) to that of the presupplementation plasma δ^{13}C-DHA (−27.6 ± 0.3 mUr). This does not mean that DHA from the supplement was not being incorporated into plasma lipids, but rather indicates that the δ^{13}C-DHA of the baseline plasma and the supplement were too similar to detect this incorporation. This is an important limitation of our study; as a secondary analysis of a randomized control trial, the δ^{13}C of the n–3 PUFA supplements were not specifically designed to contain appreciably different δ^{13}C-18:1n−9, δ^{13}C-EPA, or δ^{13}C-DHA content compared with endogenous human plasma. Future studies should be designed to contain fatty acids with δ^{13}C that are sufficiently different from the general food supply and human plasma δ^{13}C concentrations.

Previously, δ^{13}C for serum 16:0, 16:1n–7, 18:0, 18:1n–9, LA, and AA were determined before and after humans consumed a high, medium, or low saturated fatty acid diet for 8 wk (54). Serum fatty acid δ^{13}C shifted from baseline concentrations at the beginning of week 5 of diet consumption towards resemblance of dietary δ^{13}C for 16:0, 18:0, 18:1n–9, and AA. Natural variations in the δ^{13}C content of the food supply has previously been used to assess n–6 PUFA metabolism, particularly for the conversion of LA to AA in newborn infants (10, 11). In one study, infants were switched to a corn oil formula–based diet with high δ^{13}C-LA (−16.4 mUr) (10), and after only 4 d δ^{13}C increased from −31.5 to −18.8 mUr in serum LA and from −30.1 to −27.4 in serum AA. As a result, it was estimated that 23% of serum AA originated from the elongation and desaturation of LA and demonstrated that the natural variations in δ^{13}C of the food supply can not only be used for the determination of the dietary origin of blood fatty acids, but supplementation of metabolic precursors could also be used as a tool for the assessment of fatty acid metabolism in humans.

In conclusion, our study assessed the changes of plasma δ^{13}C-n–3 PUFA in humans in response to dietary supplementation, specifically EPA and DHA supplementation. Our study clearly demonstrates that changes in dietary n–3 PUFA intake can be detected in plasma δ^{13}C-n–3 PUFA and could therefore be a useful biomarker for fish and seafood intakes in various populations. We have also provided strong evidence against previously believed concepts relating to the function of the n–3 PUFA biosynthesis pathway. These new findings suggest that: *1*) there is substantial synthesis of DHA from EPA in humans, and *2*) the increases in EPA upon DHA feeding are the result of slowed EPA metabolism, and not retroconversion of DHA. Determining δ^{13}C of n–3 PUFA in humans is a remarkably powerful tool that can not only track dietary intake patterns, but can also provide novel insights into complex metabolic questions.

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