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- [AQ1] s (plus/minus) SEM correct in the Abstract Results, as inserted? Please confirm or correct.
- [AQ2] Per journal style, “level” was changed to “amount” or “concentration” as applicable throughout the article. Please confirm or amend.
- [AQ3] Journal style discourages the use of “and/or.” Please amend each instance throughout the article to either “and” or “or” as applicable.
- [AQ4] Please confirm “1.8 g EPA/d plus 1.4 g DHA ethyl ester/d” is correct.
- [AQ5] Per journals style, the unit of measure “M” must be changed to “mol/L.” Please confirm “1 mol KOH in methanol/L” is correct as edited.
- [AQ6] Throughout the text, please amend the use of “/” in such instances as “ $^{13}\text{C}/^{12}\text{C}$ ” to clarify if “and” or “:” (ie, ratio) is meant.
- [AQ7] Please confirm $\text{nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ is correct as edited, or amend each instance accordingly.
- [AQ8] In the Analytic methods section, “GraphPad Prism 5 software” is listed. If GraphPad Prism 5 for Windows software (version 5.03) is the same program, amend the previous listing for consistency.
- [AQ9] Because $^{13}\text{C}\text{-CO}_2$, $^{13}\text{C}\text{-DHA}$, and $^{13}\text{C}\text{-EPA}$ are defined in title page footnote 4, please define $^{13}\text{C}\text{-acetyl-CoA}$ in the footnote and at first use in the main text.
- [AQ10] For clarity, “those” was changed to “subjects” in the text “for subjects who did not reach 50%.”

Kinetics of ^{13}C -DHA before and during fish-oil supplementation in healthy older individuals^{1–3}

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ABSTRACT

Background: Docosahexaenoic acid (DHA) kinetics appear to change with intake, which is an effect that we studied in an older population by using uniformly carbon-13–labeled DHA (^{13}C -DHA).

Objective: We evaluated the influence of a fish-oil supplement over 5 mo on the kinetics of ^{13}C -DHA in older persons.

Design: Thirty-four healthy, cognitively normal participants (12 men, 22 women) aged between 52 and 90 y were recruited. Two identical kinetic studies were performed, each with the use of a single oral dose of 40 mg ^{13}C -DHA. The first kinetic study was performed before participants started taking a 5-mo supplementation that provided 1.4 g DHA/d plus 1.8 g eicosapentaenoic acid (EPA)/d (baseline); the second study was performed during the final month of supplementation (supplement). In both kinetic studies, blood and breath samples were collected ≤ 8 h and weekly over 4 wk to analyze ^{13}C enrichment.

Results: The time \times supplement interaction for ^{13}C -DHA in the plasma was not significant, but there were separate time and supplement effects ($P < 0.0001$). The area under the curve for plasma ^{13}C -DHA was 60% lower while subjects were taking the supplement than at baseline ($P < 0.0001$). The uniformly carbon-13–labeled EPA concentration was 2.6 times higher 1 d posttracer while

patients were taking the supplement than at baseline. The mean (\pm SEM) plasma ^{13}C -DHA half-life was 4.5 ± 0.4 d at baseline compared with 3.0 ± 0.2 d with the supplement ($P < 0.0001$). Compared with at-baseline, the mean whole-body half-life was 61% lower while subjects were taking the supplement. The loss of ^{13}C -DHA through β oxidation to carbon dioxide labeled with carbon-13 increased from 0.085% of dose/h at baseline to 0.208% of dose/h while subjects were taking the supplement.

Conclusions: In older persons, a supplement of 3.2 g EPA + DHA/d increased β oxidation of ^{13}C -DHA and shortened the plasma ^{13}C -DHA half-life. Therefore, when circulating concentrations of EPA and DHA are increased, more DHA is available for β oxidation. This trial was registered at clinicaltrials.gov as NCT01577004. *Am J Clin Nutr* 2014;100:1–8.

INTRODUCTION

From currently available dose-response studies, it has been generally accepted that the amount of DHA in plasma total lipids and/or phospholipids tends to level off when the intake of DHA and EPA is >1000 mg/d (1), whereas at <1000 mg/d, the DHA plasma dose-response relation is generally linear (2, 3). One explanation for the plateau effect of DHA and EPA doses >1000 mg/d is

that DHA is potentially more β oxidized, but to our knowledge, this possibility has not previously been shown experimentally. Indeed, regardless of the dietary intake of DHA and EPA, the kinetics of DHA in humans are still poorly understood.

In humans, the kinetics of DHA in plasma and its β oxidation can be evaluated by using an oral dose of uniformly carbon-13–labeled DHA (^{13}C -DHA)⁴. Plasma ^{13}C -DHA kinetics in humans were first reported more than a decade ago (4–6). In one study, a single oral dose of 250–280 mg ^{13}C -fatty acid mixture in which ^{13}C -DHA represented 44% of the total labeled fatty acids was given in the form of triglyceride (5). Two hours postdose, ^{13}C -DHA reached a maximum in plasma triglycerides in 3 healthy men (5). The apparent retroconversion of ^{13}C -DHA to uniformly carbon-13–labeled EPA (^{13}C -EPA) was estimated to be equivalent to 1.4% of the plasma concentration of ^{13}C -DHA (5). Neither β oxidation nor the ^{13}C -DHA half-life were reported in the studies (4–6). We recently reported the metabolism of a single 50-mg dose of 99% pure ^{13}C -DHA methyl ester given to 6 young and 6 older humans (7). We showed that 4 h after ^{13}C -DHA intake, older participants had a 4-fold higher ^{13}C -DHA concentration in plasma total lipids compared with that of the young participants (7). In older persons supplemented

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⁴ Abbreviations used: PDR, percentage of dose recovered; ^{12}C -DHA, uniformly carbon-12–labeled DHA; ^{13}C -CO₂, carbon dioxide labeled with carbon-13; ^{13}C -DHA, uniformly carbon-13–labeled DHA; ^{13}C -EPA, uniformly carbon-13–labeled EPA.

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with 323 mg EPA + 680 mg DHA for 3 wk, the unlabeled DHA in plasma total lipids reached a plateau 7 d earlier than in young adults (8). Therefore, the DHA homeostasis seems to be disturbed in older persons, which is a situation that could both influence risk of cognitive decline (9, 10) and, in turn, be influenced by a pre-existing cognitive decline. The objective of the current study was to evaluate the influence of fish-oil supplementation on ^{13}C -DHA kinetics in healthy, cognitively normal older persons.

SUBJECTS AND METHODS

Participants were >50 y old, which was an age chosen to represent the population that could potentially benefit from DHA intake in preventing cognitive decline and cardiovascular diseases (11, 12). All participants completed the Mini-Mental State Examination before and 4 mo after starting the supplement and were cognitively normal for their age (13). Exclusion criteria were diabetes, a cancer diagnosis in the past 6 mo, low serum albumin, liver or renal disease, uncontrolled hyperthyroidism or hypothyroidism, an autoimmune disorder, or C-reactive protein concentration >10 mg/L. Women had to be postmenopausal to be enrolled in this study. Because we previously showed that ^{13}C -DHA kinetics are not the same in *APOE3* and *APOE4* carriers (14), we excluded *APOE4* carriers from the current study. Other exclusion criteria included smoking, the use of n-3 PUFA supplements, overt heart disease or a cardiac event 6 mo before the study, and use of long-action benzodiazepines, warfarin, Coumadin, or a fibrate. At baseline, all participants reported having consumed ≤ 2 portions fatty fish/wk (eg, of salmon, herring, or sardines). At baseline, the mean

percentage of DHA in plasma total lipids was $1.6 \pm 0.5\%$, which indicated that DHA intake was ≤ 150 mg/d (15, 16). During the study, participants recorded fatty fish consumption in a logbook.

All participants gave informed written consent before starting the study. The study was approved by the Human Ethics Research Committee of the Health and Social Sciences Center–Sherbrooke University Geriatrics Institute.

Experimental design

The ^{13}C -DHA tracer used in this study was uniformly ^{13}C labeled (>98%) and of high chemical purity (99%). The ^{13}C -DHA was synthesized by using microalgae grown in the presence of ^{13}C -glucose according to the method of Le et al (17). Each dose comprised 40 mg ^{13}C -DHA methyl ester that, before use, was stored in an individual glass ampoule sealed under argon.

The design of the overall study and the 2 embedded kinetic studies is presented in **Figure 1**. There were 2 matching 28-d kinetic studies that used ^{13}C -DHA as follows: one study took place before subjects started taking the supplement (ie, baseline), and one study took place during the last month of supplementation with EPA and DHA (ie, supplement). The supplement consisted of 4×1.3 -g capsules of fish oil that provided 1.8 g EPA/d plus 1.4 g DHA ethyl ester/d (Ocean Nutrition Canada). We have shown that at least a 28-d follow-up is needed for the plasma ^{13}C -DHA concentration to return to near baseline after ^{13}C -DHA has been consumed orally (7, 14). The 28-d kinetic study started by the collection of fasting blood

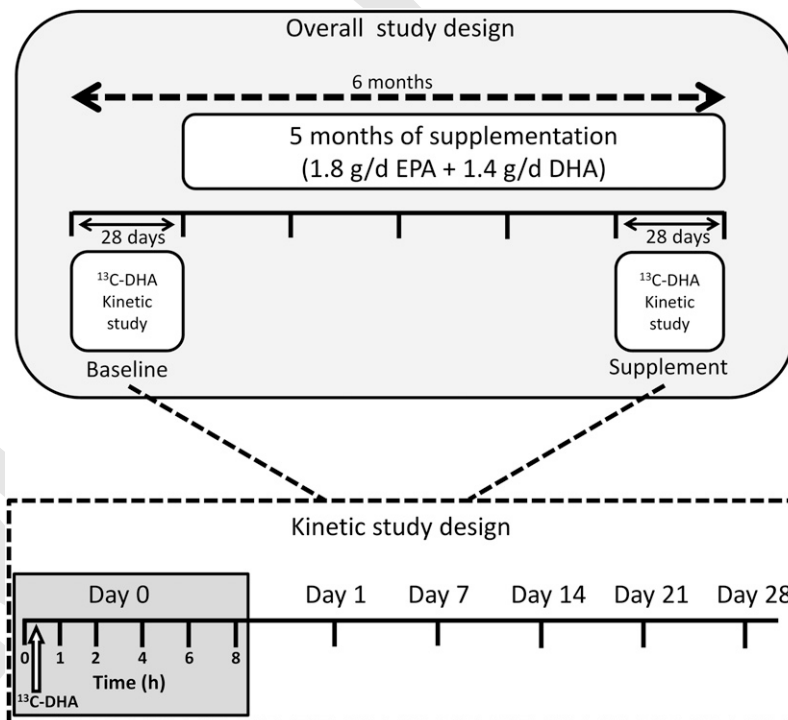


FIGURE 1. Overall study design (top) and design of the embedded kinetic studies (bottom). The kinetic studies were performed before (baseline) and in the past month of an n-3 fatty acid supplementation (supplement) providing 1.8 g/d EPA + 1.4 g/d DHA for 5 mo. In kinetic studies, a single oral dose of 40 mg ^{13}C -DHA was given to participants after collection of a fasted blood sample (time 0 h of day 0, represented by the upward-facing open arrow). The collection of blood and breath samples after ^{13}C -DHA intake was done at 1, 2, 4, 6, and 8 h of day 0, and a fasted blood and breath sample was collected at days 1, 7, 14, 21, and 28. ^{13}C -DHA, uniformly carbon-13-labeled DHA.

and breath samples (0 h), after which each participant received a single oral dose of 40 mg ¹³C-DHA deposited on a piece of toast consumed at breakfast. Breakfast comprised 2 pieces of whole-wheat toast with peanut butter, one scrambled egg, one apple, 35 g mozzarella cheese, and 250 mL orange juice. The macronutrient composition of this 670-kcal breakfast was 25.5 g fat, 78 g carbohydrate, and 29 g protein. The breakfast including the tracer was consumed by all participants within 15 min. Four hours after breakfast, participants were given a lunch consisting of store-bought lasagna with 200 mL vegetable juice and a cereal bar. The macronutrient composition of this 500-kcal lunch was 15 g fat, 88 g carbohydrate, and 23 g protein.

Blood and breath samples were collected at 0, 1, 2, 4, 6, and 8 h on day 0 and days 1, 7, 14, 21, and 28 postdose (Figure 1). A catheter was installed in each participant's forearm to collect the first 6 samples during day 0. Blood samples were collected by using a 5-mL syringe (Becton Dickinson) and transferred into 4 mL EDTA-coated tubes (Becton Dickinson). The tubes were centrifuged at 2300 × g for 15 min at 4°C, and plasma was stored in 3 0.5 mL Eppendorf tubes at -80°C until additional analysis. Alveolar breath samples at rest were collected by having subjects breath into a device that consisted of a perforated plastic bag attached to a mouthpiece (Easysampler; Quintron Instrument Co) to which an evacuated tube could be inserted to collect a sample of the exhaled breath (7, 14). These breath samples were used to follow the appearance of carbon dioxide labeled with carbon-13 (¹³C-CO₂) that came from ¹³C-DHA β oxidation.

Analytic methods

Concentrations of ¹³C-DHA and ¹³C-EPA were measured in plasma total lipids. Plasma total lipids were analyzed instead of separating by lipid classes (phospholipids, triglycerides, cholesteryl esters, and free fatty acids) because of the absence of a significant modification in concentrations of triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol while subjects were taking the supplement than at baseline (Table 1). Total lipids were extracted from 0.25 mL plasma by using Folch's method (18). Heptadecanoate was added as an internal standard to quantify fatty acids. The plasma total lipid extract was saponified by using 1 mol KOH in methanol/L and heated at 90°C for 1 h, which released fatty acids from cholesteryl esters and glycerolipids. After cooling to room temperature, hexane and saline were added. The hexane phase that contained cholesterol was discarded and the remaining saline plus fatty acid salt mixture was acidified with hydrogen chloride to obtain free fatty acids; the latter were extracted with hexane. Fatty acid methyl esters were generated by adding boron trifluoride/methanol (14%; Sigma-Aldrich) to the free fatty acids and heated at 90°C for 30 min. Fatty acid methyl esters were analyzed by using a gas chromatograph (model 6890; Agilent) equipped with a 50-m BPX-70 fused capillary column (SGE). The injection and flame-ionization detection were performed at 250°C, and the oven-temperature program was 50°C for 2 min, increased to 170°C at 20°C/min, held there for 15 min, finally increased to 210°C at 5°C/min, and held there for 7 min. Helium was the carrier gas, and the inlet pressure was 233 kPa at 50°C. Standard mixtures of fatty acids were used to identify individual fatty acids [NuChek 68A, NuChek 411, and NuChek 455 (Nu-

TABLE 1

Anthropometric characteristics and blood biochemistry of healthy older persons at baseline and while taking the supplement¹

	Baseline (n = 34)	Supplement (n = 34)	t test
Age (y)	72.4 ± 8.9 ²	—	—
Sex (M/F) (n)	12/22		
MMSE (/30) ³	28.7 ± 1.3	28.8 ± 1.3	0.635
Blood biochemistry	n = 34	n = 22	
Glucose (mmol/L)	4.7 ± 0.6	4.7 ± 0.7	0.553
Triglycerides (mmol/L)	1.4 ± 0.8	1.3 ± 0.5	0.200
Total cholesterol (mmol/L)	4.9 ± 0.8	4.8 ± 0.9	0.268
HDL cholesterol (mmol/L)	1.5 ± 0.4	1.5 ± 0.4	0.251
LDL cholesterol (mmol/L)	2.8 ± 0.7	2.8 ± 0.8	0.393
TC:HDL cholesterol	3.5 ± 0.9	3.5 ± 1.0	0.064

¹ Statistics was performed by using a paired t test. There was no significant difference (P < 0.05) while subjects were taking the supplement than at baseline for any of the blood biochemistry markers.

² Mean ± SD (all such values).

³ MMSE: Mini-Mental State Examination.

Chek Prep Inc) plus a custom mixture of SFAs]. The ¹³C-DHA enrichment in plasma total lipids was performed by using gas chromatography–combustion isotope ratio mass spectrometry as previously described (19). ¹³C/¹²C values at each time were compared with ¹³C/¹²C at baseline (t = 0 of day 0) to calculate the δ per mil values, which were designated thereafter as the atom percent excess. The Actual ¹³C enrichment in plasma DHA and EPA was calculated from atom percent excess values according to Brossard et al (5).

The enrichment of ¹³C in expired-breath CO₂ was analyzed by using isotope ratio mass spectrometry (Europa 20–20; Sercon Ltd) as previously described (20). Helium (Praxair) was the carrier gas, and 5% CO₂/N₂ was used as the reference gas. The ratio of ¹³C:¹²C-CO₂ was used to calculate the β-oxidation of ¹³C-DHA recovered in the breath in the form of ¹³C-CO₂. The percentage of dose of ¹³C-DHA recovered in the breath as ¹³C-CO₂ was calculated as previously described (21) except that basal metabolism was evaluated by using indirect calorimetry (CCM/D; Medgraphics Corp) to measure the volume of carbon dioxide (CO₂) and O₂ exhaled by participants over 30 min as previously described (22). The cumulative ¹³C-DHA β-oxidation was calculated as the AUC of the percentage of dose recovered (PDR) at each time point (GraphPad Prism 5 software; GraphPad). Specific activities of plasma DHA were calculated by using the ratio of ¹³C-DHA (nmol/mL plasma) to uniformly carbon-12-labeled DHA (¹²C-DHA; nmol/mL plasma) times 100.

The ¹³C-DHA half-life in plasma was calculated as previously described (14). The whole-body ¹³C-DHA half-life was estimated by using cumulative β-oxidation curves from days 1–28 postdose (14). For some participants, a cumulative β-oxidation of 50% was not reached within 28 d, and thus in those cases, it was assumed that cumulative ¹³C-CO₂ data >28 d would be linear (see Figure 4). With the use of cumulative ¹³C-CO₂ data at time points 1, 7, 14, 21, and 28 d postdose, a linear equation was calculated for each participant to estimate the whole-body half-life defined as the time needed to reach a 50% cumulative β-oxidation of the ¹³C-DHA dose recovered as ¹³C-CO₂. Correlations between plasma ¹³C-DHA and the percentage of dose of ¹³C-DHA recovered as breath ¹³C-CO₂ were performed for each

participant by using hierarchical linear models (measured within persons). The slope of the correlation between plasma ^{13}C -DHA and the percentage of dose of ^{13}C -DHA recovered as breath ^{13}C - CO_2 were calculated and compared between baseline and while subjects were taking the supplement.

Statistics

To calculate the sample size, we used the AUC of plasma ^{13}C -DHA as the primary endpoint. From a previous published article that used ^{13}C -DHA in older person, the mean AUC was $230 \pm 63 \text{ nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ (7). We did not have data to estimate the AUC while subjects were taking the supplement, and thus, we set the effect size at 0.5, and the δ SD was set at $63 \text{ nmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ with the assumption that the correlation (r) of the AUC at baseline compared with when subject were taking the supplement was equal to 0.5, which was conservative. We also set the power at 80% by using a paired t test with a 0.05 two-sided significance. With the use of these data, the number of participants was $n = 28$, but we anticipated a dropout of 20%, and thus, the number of participants recruited was $n = 34$. During the study, there were no dropouts. See supplemental Figure 1 under "Supplemental data" in the online issue for a presentation of a Consolidated Standards of Reporting Trials–like flowchart that shows the flow of data through the kinetic studies.

All data from plasma and breath are presented as means \pm SEMs. Data from blood and breath samples collected during kinetic studies were analyzed by using the PROC MIXED procedure implemented in SAS 9.2 software (SAS) (14). Instead of a classical 2-factor ANOVA, the PROC MIXED procedure was used to optimize the use of all data over time and maintain statistical power. This procedure allowed for the testing of the effect of time as a repeated measure, supplementation as a fixed factor (baseline compared with supplement), and the interaction time \times supplement. Student paired t tests were performed for plasma and whole-body half-lives and to detect significant differences in anthropometric characteristics and the blood biochemistry of participants while they were taking the supplement than at baseline (SPSS 17.0; SPSS Inc). AUCs were calculated with GraphPad Prism 5 for Windows software (version 5.03; GraphPad). Hierarchical linear models (measured within persons) were performed with HLM for Windows v 7.0. This multilevel model addresses within-subject and between subject variability simultaneously from a pair of submodels (23). The dependent variable was the percentage of dose of ^{13}C -DHA recovered as ^{13}C - CO_2 in the breath per hour. Independent variables were ^{13}C -DHA in plasma and the supplement. See supplemental Text under "Supplemental data" in the online issue for a presentation of the mathematical modeling. Statistical significance was set at $P \leq 0.05$.

RESULTS

Participants

There were 12 men and 22 women with an age range of 52–90 y in the study (Table 1). Their mean (\pm SEM) score on the Mini-Mental State Examination was 29 ± 1 both at baseline and while subjects were taking the supplement, which confirming that subjects were cognitively normal throughout the study. Blood biochemistry was

performed on 34 subjects at baseline and 22 subjects while they were taking the supplement. Initially, it was intended that blood biochemistry be performed to evaluate the eligibility of participants only at baseline. Thereafter, the protocol was amended to include a second evaluation of blood biochemistry while participants were taking the supplement, but the first 12 participants had already completed the study. Blood glucose, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, and total cholesterol/HDL cholesterol did not significantly change while subjects were taking the supplement than at baseline (Table 1). During the 5 mo that subject were taking the supplement, all participants reported the consumption of <2 portions fatty fish/wk. At baseline, means of plasma EPA and DHA were 117 ± 52 and $183 \pm 70 \text{ nmol/mL}$, respectively, which represented $0.95 \pm 0.38\%$ and $1.60 \pm 0.53\%$ of total fatty acids, respectively (see supplemental Table 1 under "Supplemental data" in the online issue). One month after subject started supplementation, EPA and DHA plateaued in total lipids of plasma at concentrations between 383 and 589 nmol/mL for EPA and between 290 and 376 nmol/mL for DHA (Figure 2).

^{13}C -DHA kinetics

At baseline, plasma ^{13}C -DHA peaked 6 h postdose at $2.0 \pm 0.2 \text{ nmol/mL}$, whereas it peaked 4 h postdose at $1.6 \pm 0.1 \text{ nmol/mL}$ when subjects were taking the supplement (Figure 3A). The time \times supplement interaction was not significant, but there were separate time and supplement effects ($P < 0.0001$) such that, over the follow-up period of 28 d, the mean difference of ^{13}C -DHA at each time was not significantly lower while subjects were taking the supplement than at baseline. However, the AUC of plasma ^{13}C -DHA while subjects were taking the supplement was 60% lower than at baseline ($P < 0.0001$; Figure 3A). The rising slope of plasma ^{13}C -DHA was the same at baseline as while subjects were taking the supplement (Figure 3A).

Plasma tracee (^{12}C -DHA; nmol/L) was stable over the 28-d follow-up period and was $\sim 70\%$ higher while subjects were taking the supplement than at baseline. The specific activity is presented in Figure 3B and defined as $^{13}\text{C}\text{-DHA}/^{12}\text{C}\text{-DHA}$. At baseline, the peak specific activity was $1.12 \pm 0.08\%$ compared with $0.53 \pm 0.04\%$ while subjects were taking the supplement, with a significant time \times supplement interaction ($P < 0.0001$) that showed that the kinetics of specific activity over 28 d differed significantly in participants while they were taking the

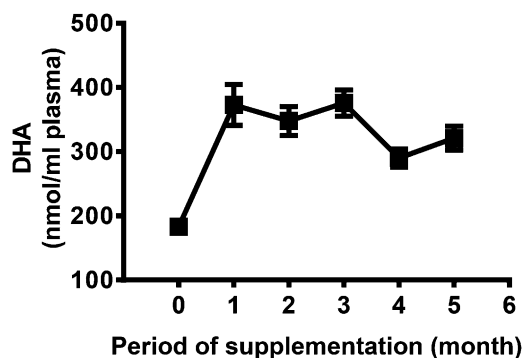
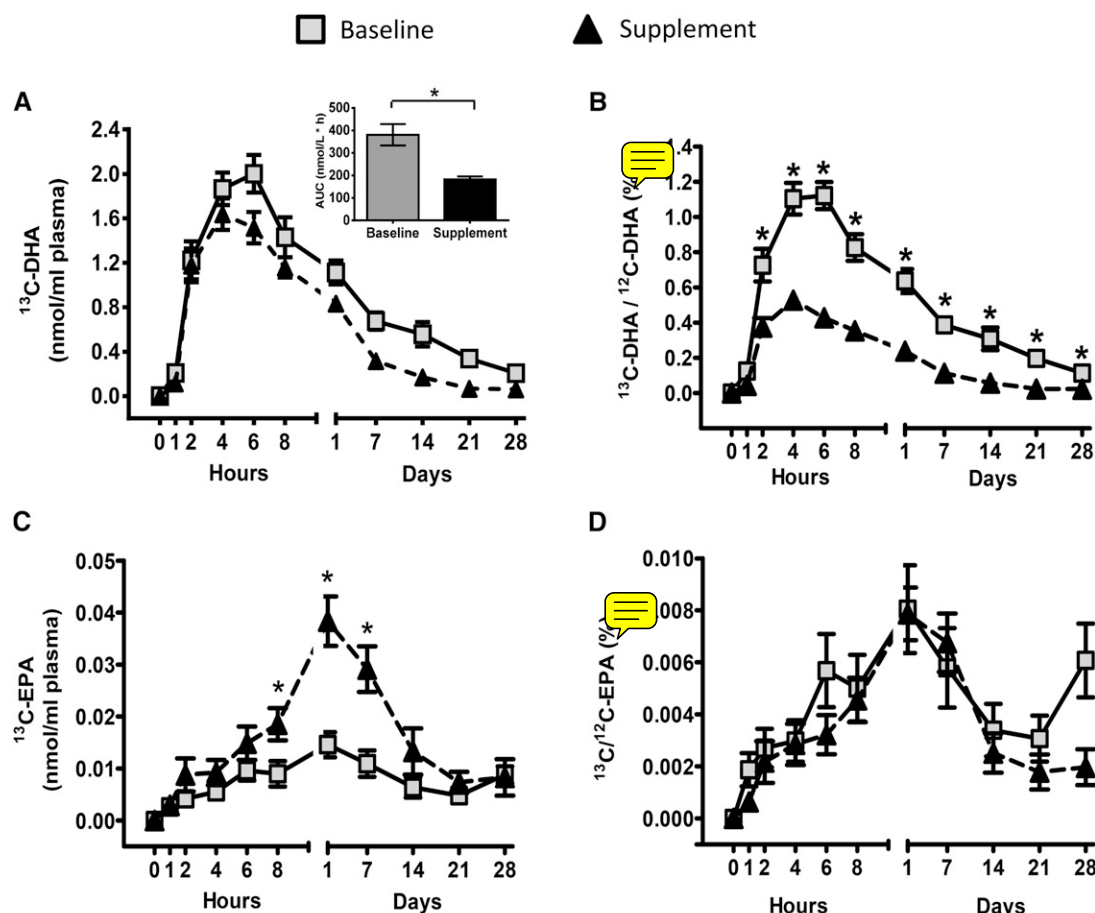


FIGURE 2. Mean (\pm SEM) DHA in plasma total lipids over the 5-mo supplementation period. One month after subjects started taking the supplement, the concentration of DHA doubled in plasma total lipids and reached a plateau ($n = 34$).



Time after ¹³C-DHA dose

FIGURE 3. Mean (\pm SEM) concentrations of ¹³C-DHA (A), ¹³C/¹²C-DHA (B), ¹³C-EPA (C) ¹³C/¹²C-EPA (D) in plasma total lipids over 28 d at baseline and while subjects were taking the supplement ($n = 34$). Statistics were performed by using the PROC MIXED procedure implemented in SAS 9.2 software (SAS) and tested the effect of time as a repeated measure, supplementation as a fixed factor (baseline compared with supplement), and the interaction of time \times supplement. When there was an interaction, comparisons at each time were performed by using a paired t test. For the AUC in A, a paired t test was performed. In A, the ¹³C-DHA concentration varies significantly over time, and there was an independent supplement effect. In B and C, there was a time \times supplement interaction. In D, ¹³C-EPA/¹²C-EPA varied significantly over time only ($*P < 0.05$). ¹²C-DHA; uniformly carbon-12-labeled DHA; ¹²C-EPA, uniformly carbon-12-labeled EPA; ¹³C-DHA, uniformly carbon-13-labeled DHA; ¹³C-EPA, uniformly carbon-13-labeled EPA.

supplement than at baseline. The specific activity was 2.5–7-times higher at baseline than while subjects were taking the supplement throughout the metabolic follow-up period 1–28 d postdose (Figure 3B).

There was a significant time \times supplement interaction for plasma ¹³C-EPA with 2.0–2.8-times higher plasma ¹³C-EPA from 8 h to 7 d postdose while subjects were taking the supplement than at baseline (Figure 3C). The specific activity of plasma ¹³C-EPA was significantly different over time ($P < 0.0001$), but there was no supplement effect and no interaction (Figure 3D).

The cumulative β -oxidation of ¹³C-DHA to ¹³C-CO₂ over 28 d was 1.9-times higher while subjects were taking the supplement than at baseline (Figure 4). The peak ¹³C-CO₂ enrichment occurred 4 h postdose and reached 0.48% of dose of ¹³C-DHA/h while subjects were taking the supplement compared with 0.31% of dose per hour at baseline (data not shown). The enrichment of ¹³C-CO₂ returned almost to baseline 28 d postdose at baseline and while subjects were taking the supplement (data not shown). The cumulative β -oxidation at 28 d was 35.1 \pm

6.7% of the tracer dose at baseline compared with 65.7 \pm 9.1% while subjects were taking the supplement (Figure 4).

DHA half-life

The mean plasma half-life of ¹³C-DHA was 4.5 \pm 0.4 d at baseline compared with 3.0 \pm 0.2 d while subjects were taking the supplement ($P < 0.0001$; Figure 5A). The mean whole-body half-life of ¹³C-DHA was 140 \pm 27 d at baseline compared with 54 \pm 12 d while subjects were taking the supplement ($P = 0.0107$; Figure 5B). Neither the plasma nor whole-body ¹³C-DHA half-life differed between men and women, and there was not a significant correlation with the age of participants.

Hierarchical linear model

The final estimation of the HLM model is shown in Figure 6. At baseline, ¹³C-DHA was positively related to PDR as ¹³C-CO₂ with a slope of 0.0847, whereas while subjects were taking the supplement, the slope was much steeper at 0.2076 ($P < 0.001$), which showed that, for a similar concentration of ¹³C-DHA in

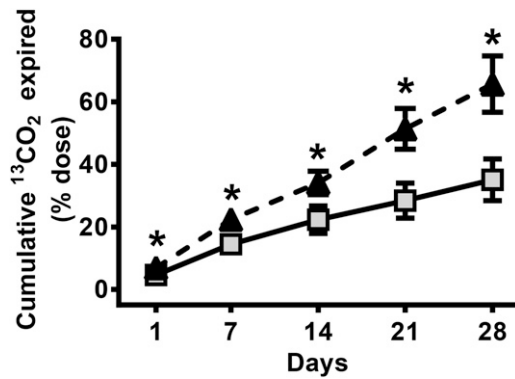


FIGURE 4. Mean (\pm SEM) cumulative percentage of dose of ^{13}C -DHA β -oxidized and recovered in the breath as ^{13}C - CO_2 at baseline (gray squares) and while subjects were taking the supplement (black triangles) ($n = 34$). Statistics were performed using a PROC MIXED procedure implemented SAS 9.2 software (SAS) and tested the effect of time as a repeated measure, supplementation as a fixed factor (baseline compared with supplement), and the interaction of time \times supplement. When there was an interaction, comparisons at each time point were performed by using a paired t test. There was a time \times supplement interaction supporting that the difference in cumulative ^{13}C - CO_2 expired while subjects were taking the supplement; baseline was not the same at each time point ($*P < 0.05$). ^{13}C - CO_2 , carbon dioxide labeled with carbon-13; ^{13}C -DHA, uniformly carbon-13-labeled DHA.

blood, the β -oxidation of ^{13}C -DHA to ^{13}C - CO_2 was higher while subjects were taking the supplement than at baseline. Plasma ^{13}C -DHA explained 50% of the PDR variation in the model. The intersubject variation (u_i) associated with random effect (β_{kj}) was tested by using age, sex, education, and BMI as covariates (*see* supplemental Text for a description of the mathematical model under “Supplemental data” in the online issue). BMI was the only covariate to be significantly involved in explaining the intercept and contributed to $\sim 5\%$ of the variation in the relation between PDR and plasma ^{13}C -DHA.

DISCUSSION

In this study, we report that supplementation with an EPA + DHA supplement significantly modified the kinetics of ^{13}C -DHA in healthy older persons without cognitive decline such that its plasma and whole-body half-lives were lower and its β -oxidation to ^{13}C - CO_2 was higher than at baseline. Hence, the plateauing of plasma DHA at higher intakes of EPA + DHA appears to be a function of increased β -oxidation.

Despite the higher β -oxidation of DHA while subjects were taking the supplement, the cumulative β -oxidation of ^{13}C -DHA remained low compared with what has typically been reported for other long-chain fatty acids. For example, 24 h postdose, the β -oxidation of ^{13}C -DHA was $<7\%$ while subjects were taking the supplement and at baseline (Figure 4), whereas the β -oxidation over the same period and under essentially the same conditions was 29% for ^{13}C -oleic acid, 21% for ^{13}C -linoleic acid, and 31% for ^{13}C - α -linolenic acid (20). These results were potentially because, in contrast to PUFAs with <20 carbons, PUFAs with >20 carbons require the previous peroxisomal β -oxidation to shorten the fatty acid chain before entry to the mitochondrial β oxidation spiral (24, 25). However, the relative contribution of peroxisomal compared with mitochondrial β -oxidation to the whole-body production of ^{13}C - CO_2 from ^{13}C -DHA in humans is unknown. The affinity of carnitine palmitoyl

transferase is also much lower for DHA than long chain fatty acids with 1–3 double bonds (26). Thus, DHA is relatively efficiently conserved, probably because of its structural importance in cell membranes (27) and as a precursor to signaling molecules (28).

Our ^{13}C - CO_2 breath results did not account for the ^{13}C that was converted to ^{13}C -acetyl-CoA but then recovered in other metabolites rather than entering the Krebs' cycle and being converted to ^{13}C - CO_2 . However, during our gas chromatography-combustion isotope ratio mass spectrometry analysis, there was no measurable ^{13}C enrichment in any other plasma fatty acids except EPA, which suggested that, once ^{13}C -DHA starts to be β -oxidized, the process goes mostly to completion (ie, quantitatively to ^{13}C - CO_2).

The whole-body half-life was calculated from the ^{13}C - CO_2 recovery in the breath to estimate the time needed to β -oxidize 50% of the ^{13}C -DHA dose without regard to compartments. In this calculation, it was assumed that the quantity of ^{13}C - CO_2 going from peripheral compartments to plasma and then to breath remained constant after the 28th d for subjects who did

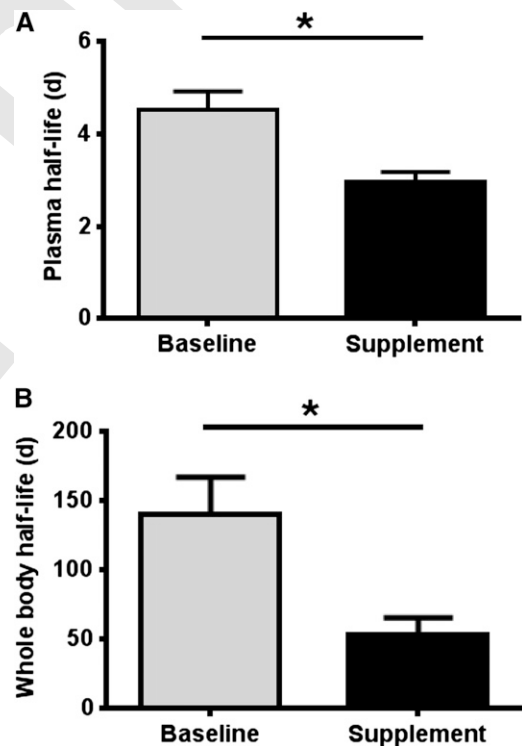


FIGURE 5. Mean (\pm SEM) calculated half-life of ^{13}C -DHA at baseline and while subjects were taking the supplement in plasma total lipids ($n = 32$ at baseline and $n = 34$ while subjects were taking the supplement) (A) and the whole body ($n = 29$ at baseline and $n = 32$ while subjects were taking the supplement) (B). Paired t tests were performed for comparison of while subjects were taking the supplement with at baseline. At baseline, blood samples collected at 14, 21, and/or 28 d because 2 participants had an enrichment of ^{13}C over ^{12}C below the enrichment at 0 h of day 0, and thus, it was not possible to calculate the plasma half-life in these participants. In 5 participants at baseline and 2 participants while taking the supplement, the cumulative β -oxidation of ^{13}C -DHA recovered as carbon dioxide labeled with carbon-13 reached a plateau of $<50\%$ 7 d postdose, and thus, the calculation of the whole-body ^{13}C -DHA half-life was not possible. While subjects were taking the supplement, plasma and whole-body half-lives were 33–61% lower compared with at baseline. $*P \leq 0.05$. ^{13}C -DHA, uniformly carbon-13-labeled DHA.

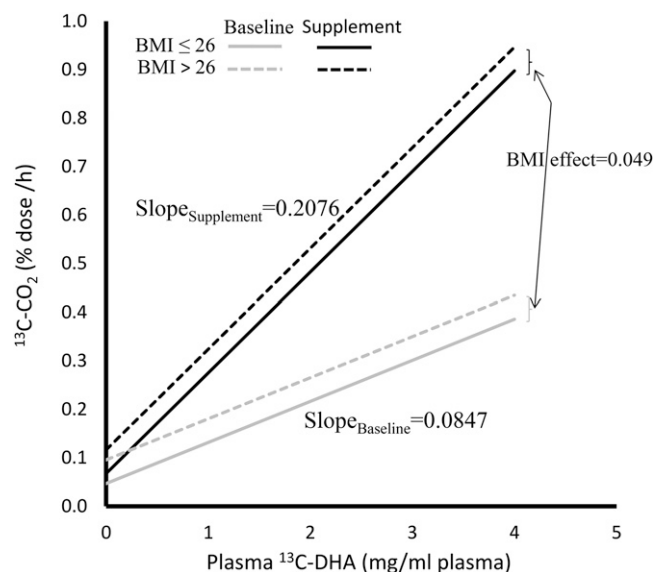


FIGURE 6. Mean (\pm SEM) correlations between ¹³C-CO₂ and plasma ¹³C-DHA concentrations at baseline and while subjects were taking the supplement separated by the median BMI (in kg/m²) ≤ 26 or > 26 . The Hierarchical linear model test was used to plot mean correlation curves from individual curves of 34 participants (see supplemental Text under “Supplemental data” in the online issue for the description of the mathematical model). The slope of curves was significantly different while subjects were taking the supplement than at baseline, which suggested that, for a similar concentration of ¹³C-DHA in blood, the β -oxidation of ¹³C-DHA expressed as the percentage of dose of ¹³C-DHA recovered as ¹³C-CO₂/h was higher while subjects were taking the supplement than at baseline. BMI significantly explained $\sim 5\%$ of the variation in the relation between the percentage of dose of ¹³C-DHA recovered as ¹³C-CO₂/h and plasma ¹³C-DHA. ¹³C-CO₂, carbon dioxide labeled with carbon-13; ¹³C-DHA, uniformly carbon-13-labeled DHA.

not reach 50% of cumulative ¹³C-CO₂. Hence, the calculated whole-body half-life represents the best estimate of the time needed for one-half of the ¹³C-DHA dose to be exhaled in the form of ¹³C-CO₂ by participants, with the recognition that this is probably an underestimate because some β -oxidized ¹³C-DHA could theoretically get incorporated into other compounds synthesized via ¹³C-acetyl-CoA.

The lower postprandial specific activity of plasma ¹³C-DHA while subjects were taking the supplement can largely be explained by the 70% tracer dilution compared with at baseline (Figure 3B). ¹³C-DHA kinetics may also have been affected because the supplement was not pure DHA but rather a combination of EPA + DHA. The provision of pure DHA results in an apparently linear increase in EPA in plasma phospholipids (16). Therefore, if the supplement had been pure DHA, it is possible that the ¹³C-EPA concentration in plasma would have been higher than we observed, but this possibility is necessarily speculative. Similarly, had the supplement provided a dose of EPA + DHA closer to the international recommendation (rather than 6 times above the recommendation as was the case in this study), the effect on ¹³C-DHA kinetics would likely have been attenuated.

Unlike the specific activity of plasma ¹³C-DHA, the specific activity of plasma ¹³C-EPA was not significantly lower while subjects were taking the supplement than at baseline (Figure 3D) despite the EPA + DHA supplement having raised plasma EPA 5-fold. Changes in the mass of ¹³C-EPA during EPA + DHA

supplementation may originate via several routes, including the retroconversion from DHA, carbon recycling that provides ¹³C-acetyl-CoA for stearidonic acid elongation, and/or the sparing of EPA from β -oxidation. No change in the specific activity of plasma EPA suggested that EPA sparing was significant while subjects were taking the supplement. Because of the bigger pool of circulating ¹³C-DHA, the apparent rate of retroconversion to EPA or displacement of EPA from nonplasma pools appears to have been unchanged by the supplement. In contrast, the slope of ¹³C-DHA β -oxidation increased with higher circulating DHA while participants were taking the supplement than at baseline (Figure 6). These data suggest that the partitioning of DHA between β -oxidation and retroconversion to EPA are probably regulated separately.

In the current study, there was no difference in plasma ¹³C-DHA, plasma DHA, or the ¹³C-DHA half-life in whole body or plasma between men and women (data not shown). The absence of sex-specific differences was probably because of the drop in estrogen in women after menopause (29). Two other factors that potentially change DHA homeostasis are age and APOE4 genotype (7, 14). APOE4 carriers were excluded from this study (15). Age-dependent differences in ¹³C-DHA metabolism have been reported elsewhere over a 50-y difference in age (7). In the current study, there was no correlation between age (52–90 y old) and the ¹³C-DHA half-life, but this study was not designed to assess a possible age effect. Participants in this study were of an age when cognitive decline could be starting, but they were all cognitively healthy as assessed by using the Mini-Mental State Examination. This fact is important because we wanted to eliminate lower cognitive performance as a confounder that might have influenced the results (10).

In conclusion, in older persons taking 3.2 g EPA + DHA/d, there is an increased β -oxidation of ¹³C-DHA and shortened plasma and whole-body ¹³C-DHA half-life. Therefore, when circulating concentrations of EPA + DHA are increased, more DHA appears to be available for β oxidation.

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The authors’ responsibilities were as follows—MP and SCC: designed the research; RC-W, MP, MF, CR-P, JT-M, MTMD, M-JA, and PP: conducted experiments and collected data; MP, RC-W, JTB, and SCC: analyzed data or performed the statistical analysis; MP, JTB, and SCC: wrote the manuscript; MP: had primary responsibility for the final content the manuscript; YZ, PL, and JTB: performed the ¹³C-fatty acid analysis; DL: conducted the cognitive testing; M-CV: performed the genotyping; and all authors: read and approved the final version of the manuscript. None of the authors had a conflict of interest.

REFERENCES

- Blonk MC, Bilo HJ, Nauta JJ, Popp-Snijders C, Mulder C, Donker AJ. Dose-response effects of fish-oil supplementation in healthy volunteers. *Am J Clin Nutr* 1990;52:120–7.
- Cunnane SC, Chouinard-Watkins R, Castellano CA, Barberger-Gateau P. Docosahexaenoic acid homeostasis, brain aging and Alzheimer’s disease: can we reconcile the evidence? *Prostaglandins Leukot Essent Fatty Acids* 2013;88:61–70.
- Arterburn LM, Oken HA, Hoffman JP, Bailey-Hall E, Chung G, Rom D, Hamersley J, McCarthy D. Bioequivalence of docosahexaenoic acid from different algal oils in capsules and in a DHA-fortified food. *Lipids* 2007;42:1011–24.

4. Brenna JT. Use of stable isotopes to study fatty acid and lipoprotein metabolism in man. *Prostaglandins Leukot Essent Fatty Acids* 1997; 57:467–72.
5. Brossard N, Croset M, Pachiardi C, Riou JP, Tayot JL, Lagarde M. Retroconversion and metabolism of [^{13}C]22:6n–3 in humans and rats after intake of a single dose of [^{13}C]22:6n–3-triacylglycerols. *Am J Clin Nutr* 1996;64:577–86.
6. Lemaitre-Delaunay D, Pachiardi C, Laville M, Pousin J, Armstrong M, Lagarde M. Blood compartmental metabolism of docosahexaenoic acid (DHA) in humans after ingestion of a single dose of [^{13}C]DHA in phosphatidylcholine. *J Lipid Res* 1999;40:1867–74.
7. Plourde M, Chouinard-Watkins R, Vandal M, Zhang Y, Lawrence P, Brenna JT, Cunnane SC. Plasma incorporation, apparent retroconversion and beta-oxidation of ^{13}C -docosahexaenoic acid in the elderly. *Nutr Metab (Lond)* 2011;8:5.
8. Vandal M, Freemantle E, Tremblay-Mercier J, Plourde M, Fortier M, Bruneau J, Gagnon J, Tremblay S, Bégin M, Cunnane SC. Plasma omega-3 fatty acid response to a fish oil supplement in the healthy elderly. *Lipids* 2008;43:1085–9.
9. Schaefer EJ, Bongard V, Beiser AS, Lamou-Fava S, Robins SJ, Au R, Tucker KL, Kyle DJ, Wilson PW, Wolf PA. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. *Arch Neurol* 2006;63:1545–50.
10. Cunnane SC, Schneider JA, Tangney C, Tremblay-Mercier J, Fortier M, Bennett DA, Morris MC. Plasma and brain fatty acid profiles in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis* 2012;29:691–7.
11. Yurko-Mauro K, McCarthy D, Rom D, Nelson EB, Ryan AS, Blackwell A, Salem N Jr, Stedman M. Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline. *Alzheimers Dement* 2010;6:456–64.
12. Yurko-Mauro K. Cognitive and cardiovascular benefits of docosahexaenoic acid in aging and cognitive decline. *Curr Alzheimer Res* 2010;7: 190–6.
13. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975;12:189–98.
14. Chouinard-Watkins R, Rioux-Perreault C, Fortier M, Tremblay-Mercier J, Zhang Y, Lawrence P, Vohl MC, Perron P, Lorrain D, Brenna JT, et al. Disturbance in uniformly ^{13}C -labelled DHA metabolism in elderly human subjects carrying the apoE epsilon4 allele. *Br J Nutr* 2013;110:1751–9.
15. Hennebelle M, Plourde M, Chouinard-Watkins R, Castellano CA, Barberger-Gateau P, Cunnane SC. Ageing and apoE change DHA homeostasis: relevance to age-related cognitive decline. *The Proc Nutr Soc* 2014;73:80–6.
16. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n–3 fatty acids in humans. *Am J Clin Nutr* 2006;83: 1467S–76S.
17. Le PM, Fraser C, Gardner G, Liang WW, Kralovec JA, Cunnane SC, Windust AJ. Biosynthetic production of universally (^{13}C)-labelled polyunsaturated fatty acids as reference materials for natural health product research. *Anal Bioanal Chem* 2007;389:241–9.
18. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957; 226:497–509.
19. Goodman KJ, Brenna JT. High sensitivity tracer detection using high-precision gas chromatography-combustion isotope ratio mass spectrometry and highly enriched [^{13}C]-labeled precursors. *Anal Chem* 1992;64:1088–95.
20. McCloy U, Ryan MA, Pencharz PB, Ross RJ, Cunnane SC. A comparison of the metabolism of eighteen-carbon ^{13}C -unsaturated fatty acids in healthy women. *J Lipid Res* 2004;45:474–85.
21. Freemantle E, Vandal M, Tremblay Mercier J, Plourde M, Poirier J, Cunnane SC. Metabolic response to a ketogenic breakfast in the healthy elderly. *J Nutr Health Aging* 2009;13:293–8.
22. Choquette S, Chuin A, Lalancette DA, Brochu M, Dionne IJ. Predicting energy expenditure in elders with the metabolic cost of activities. *Med Sci Sports Exerc* 2009;41:1915–20.
23. Raudenbush S, Bryk A. Hierarchical linear models: application and data analysis methods. 2nd ed. Thousand Oaks, CA: Sage Publications, 2002.
24. Sprecher H. Metabolism of highly unsaturated n–3 and n–6 fatty acids. *Biochim Biophys Acta* 2000;1486:219–31.
25. Jump DB. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol* 2002;13:155–64.
26. Gavino GR, Gavino VC. Rat liver outer mitochondrial carnitine palmitoyltransferase activity towards long-chain polyunsaturated fatty acids and their CoA esters. *Lipids* 1991;26:266–70.
27. Litman BJ, Niu SL, Polozova A, Mitchell DC. The role of docosahexaenoic acid containing phospholipids in modulating G protein-coupled signaling pathways: visual transduction. *J Mol Neurosci* 2001; 16:237–42; discussion 279–84.
28. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J Exp Med* 2000;192:1197–204.
29. Cauley JA, Gutai JP, Kuller LH, LeDonne D, Powell JG. The epidemiology of serum sex hormones in postmenopausal women. *Am J Epidemiol* 1989;129:1120–31.