Effect of altered dietary *n*-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [^{13}C]α-linolenic acid to longer-chain fatty acids and partitioning towards β-oxidation in older men

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The effect of increased dietary intakes of α-linolenic acid (ALNA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for 2 months upon plasma lipid composition and capacity for conversion of ALNA to longer-chain metabolites was investigated in healthy men (52 (SD 12) years). After a 4-week baseline period when the subjects substituted a control spread, a test meal containing [U-^{13}C]ALNA (700 mg) was consumed to measure conversion to EPA, docosapentaenoic acid (DPA) and DHA over 48 h. Subjects were then randomised to one of three groups for 8 weeks before repeating the tracer study: (1) continued on same intake (control, *n* 5); (2) increased ALNA intake (10 g/d, *n* 4); (3) increased EPA+DHA intake (1·5 g/d, *n* 5). At baseline, apparent fractional conversion of labelled ALNA was: EPA 2·80, DPA 1·20 and DHA 0·04 %. After 8 weeks on the control diet, plasma lipid composition and [^{13}C]ALNA conversion remained unchanged compared with baseline. The high-ALNA diet resulted in raised plasma triacylglycerol-EPA and -DPA concentrations and phosphatidylcholine-EPA concentration, whilst [^{13}C]ALNA conversion was similar to baseline. The high-(EPA+DHA) diet raised plasma phosphatidylcholine-EPA and -DHA concentrations, decreased [^{13}C]ALNA conversion to EPA (2-fold) and DPA (4-fold), whilst [^{13}C]ALNA conversion to DHA was unchanged. The dietary interventions did not alter partitioning of ALNA towards β-oxidation. The present results indicate ALNA conversion was down-regulated by increased product (EPA+DHA) availability, but was not up-regulated by increased substrate (ALNA) consumption. This suggests regulation of ALNA conversion may limit the influence of variations in dietary *n*-3 fatty acid intake on plasma lipid compositions.

α-Linolenic acid: Stable isotope: Dietary *n*-3 polyunsaturated fatty acids: Man

The long-chain polyunsaturated fatty acids (FA) eicosapentaenoic (20 : 5*n*-3, EPA) and docosahexaenoic acids (22 : 6*n*-3, DHA) have beneficial effects in reducing the risk of cardiovascular disease (Simoupolos, 1997). Despite recommendations that consumption of oily fish should increase (Committee on Medical Aspects of Food Policy, 1994; British Nutrition Foundation, 1999; Simopolous *et al.* 1999). The efficacy of this strategy as a means of increasing EPA and DHA intakes in the general population is limited, at least in part, by the unpalatability of fish to many individuals (Gregory *et al.* 1990). The precursor α-linolenic acid (18 : 3*n*-3, ALNA) is the major *n*-3 FA in the western diet (Ministry of Agriculture, Fisheries and Food, 1997; Kris-Etherton *et al.* 2000), while consumption

of pre-formed EPA and DHA is about 10-fold lower. Thus, maintenance of tissue EPA and DHA concentrations may depend, at least in part, upon metabolic capacity for ALNA conversion. This suggests the possibility that increased ALNA intake may represent an alternative to raising oily fish consumption as a strategy for increasing tissue EPA and DHA concentrations.

A pathway for conversion of ALNA to EPA and DHA has been demonstrated in the rat. Such conversion involves sequential desaturation and C-chain elongation to form EPA and docosapentaenoic acid (22 : 5*n*-3, DPA). DHA synthesis from DPA is the result of further chain elongation and desaturation to form 24 : 6*n*-3, followed by limited peroxisomal β-oxidation (Sprecher, 2000).

While the conversion of ALNA to 18 : 4*n*-3 by Δ6-desaturase is the principal rate-limiting step of the pathway, the peroxisomal β-oxidation step may also act as an important locus of metabolic regulation (Sprecher, 2000).

The few studies that have estimated the extent of ALNA conversion in adult human subjects show that the apparent conversion to EPA is limited (<8 %) while DHA synthesis is marginal ($<0.02-4.00$ %) (Emken *et al.* 1994; Salem *et al.* 1999; Vermunt *et al.* 1999, 2000; Pawlosky *et al.* 2001; Burdge *et al.* 2002). The extent to which dietary intake of *n*-3 long-chain polyunsaturated FA influences conversion of ALNA to longer-chain FA in man has not been characterised fully. Two studies have shown increased EPA and/or DHA consumption to decrease the conversion of labelled ALNA to both EPA and DHA (Emken *et al.* 1999; Vermunt *et al.* 2000). Together, these results suggest feedback inhibition of ALNA conversion due to accumulation of product. Increasing ALNA intake was shown to decrease synthesis of EPA by about 30 %, but did not alter DPA and DHA synthesis (Vermunt *et al.* 2000). The mechanism by which increased ALNA consumption decreased the activity of the pathway is unclear, although competition between dietary linoleate and ALNA for Δ6-desaturase has been suggested as a possible explanation (Vermunt *et al.* 2000). The observation of reduced ALNA conversion with increased ALNA intakes is in contrast with reports that demonstrate that dietary supplementation of ALNA increase plasma or membrane EPA and/or DPA concentrations (Kwon *et al.* 1991; Chan *et al.* 1993; Freese *et al.* 1994; Allman *et al.* 1995; Cunnane *et al.* 1995; Mantzioris *et al.* 1995; Li *et al.* 1999; Finnegan *et al.* 2003). While the majority of these studies did not show an increase in plasma or membrane DHA concentration with increased ALNA intakes, two reports have indicated that supplementation with ALNA was associated with an increase in DHA concentration (Beitz *et al.* 1981; Ezaki *et al.* 1999). Overall, the FA composition results suggest increased ALNA consumption may up-regulate ALNA conversion to longer-chain FA, but the extent of conversion to longer-chain products remains uncertain. Therefore, there is a need to resolve the relationship between the ALNA and EPA+DHA content of the diet and metabolic capacity for conversion of ALNA to longer-chain FA using techniques which measure synthesis of longer-chain FA in addition to tissue accumulation.

In the present study, we have tested the hypothesis that the extent of ALNA conversion to long-chain products is up-regulated by increased dietary ALNA intake (substrate), but is decreased by enriching the diet with EPA and DHA (product), and that the magnitude of such effects exceeds any temporal variation in ALNA desaturation and/or elongation that occurs on a standard diet. We report the effect of increasing either dietary ALNA or EPA+DHA intakes for 8 weeks on plasma lipid compositions and metabolic conversion of [U-^{13}C]ALNA in men participating in a larger study of the effect of *n*-3 FA supplementation on risk factors for cardiovascular disease (Finnegan *et al.* 2003). It is important to establish the metabolic capacity of older individuals, since it is this group at whom recommendations for change in dietary *n*-3 polyunsaturated FA consumption are directed. We describe the longitudinal effect of these dietary interventions on partitioning of [^{13}C]ALNA between β-oxidation and conversion to longer-chain FA, and the concentrations of labelled *n*-3 FA in plasma lipids over 48 h by comparing the results of tracer studies carried out before and at the end of the dietary intervention.

Experimental procedures

Materials

Solvents were purchased from Fisher Chemicals Ltd (Loughborough, Leics., UK). Lipid standards were from Sigma (Poole, Dorset, UK). [U-^{13}C]ALNA (^{13}C enrichment >98 atom %) was obtained from Martek Biosciences Corporation (Columbia, MD, USA). Our analysis showed this preparation contained 97·1 g ALNA/100 g total FA, the remainder being FA of chain length $<C_{12}$. The novel spreads were prepared by Unilever Health Institute (Vlaardingen, The Netherlands) and the oil capsules were prepared by Roche Vitamins Limited (Basel, Switzerland).

Subjects

Healthy men (*n* 14) aged 52 (SD 12) years were recruited from participants in a larger (*n* 150) intervention trial (Finnegan *et al.* 2003). Their fasting triacylglycerol (TAG) concentration was appropriate for age (Table 1) (Ball & Mann, 1988). Fifteen subjects were recruited, of which two withdrew and one was replaced. Subject characteristics are summarised in Table 1. None of the subjects took FA or antioxidant supplements, or consumed more than two portions of oily fish per week. The study was approved by the University of Reading Ethics and Research Committee, and each volunteer gave written consent before participating.

Dietary interventions

The dietary intervention strategy is described in detail in Finnegan *et al.* (2003). The interventions were administered using a combination of fat spreads formulated specifically for the study and capsules. The first 4 weeks served as a run-in period, where all participants consumed a control spread with no additional *n*-3 polyunsaturated FA (Table 2). Subjects were then assigned to one of three dietary groups matched for age, BMI and fasting TAG concentration (Table 1): (1) continue the same FA intake as the run-in period (control group); (2) increased ALNA intake (10 g/d; high-ALNA group); (3) increased combined EPA+DHA consumption (1·5 g/d; high-(EPA+DHA) group) for a period of 8 weeks (Table 2). The target intakes were calculated to include the estimated average contribution from the background UK diet of EPA+DHA (0·2 g/d) and ALNA (1·5 g/d) (Ministry of Agriculture, Fisheries and Food, 1997).

The intervention was achieved using an allowance of 25 g novel spread/d: the subjects substituted this for their habitual spread, together with two capsules containing purified oil per d consumed with meals (Table 2). Subjects were not allowed to use the spreads for baking or frying.

Table 1. Characteristics of each intervention group after randomisation based on age, BMI and fasting triacylglycerol concentration
(Mean values and standard deviations)

Intervention group...	Control (n 5)		High-(EPA+DHA) (n 5)		High-ALNA (n 4)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	50	16	53	8	51	14
Baseline BMI (kg/m^2)	26·3	3·3	27·2	3·4	24·9	3·9
Baseline TAG (mmol/l)	1·6	0·3	1·6	0·6	1·7	0·8
Baseline total cholesterol (mmol/l)	6·0	1·3	5·5	0·9	5·0	0·2
Baseline LNA intake (mg/d)	2222	846	1766	980	1667	769
LNA intake for 5 weeks (mg/d)	1684	708	1684	708	9553*†‡	832
Baseline EPA+DHA intake (mg/d)	474	376	264	164	492	509
EPA+DHA for 5 weeks intake (mg/d)	389	132	1627*†§	328	384	260
Baseline linoleic acid intake (g/d)	17	6	19	13	16	2
Linoleic acid intake for 5 weeks (g/d)	24	4	23	7	15	4
Baseline total fat intake (g/d)	116	37	122	62	105	37
Total fat intake for 5 weeks (g/d)	122	35	132	61	116	22
Baseline energy intake (MJ/d)	12·5	2·7	12·8	4·9	12·5	2·0
Within-intervention-group energy intake (MJ/d)	12·9	3·2	13·0	5·3	12·3	1·5

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALNA, α-linolenic acid; TAG, triacylglycerol.
Mean values were significantly different from baseline values within an intervention group (Student's t test): *P<0·0001.
Mean values were significantly different from control group within a time point: (one-way ANOVA) †P<0·001.
Mean values were significantly different from high-(EPA + DHA) group (one-way ANOVA) within a time point: ‡P<0·001.
Mean values were significantly different from high-ALNA group (one-way ANOVA) within a time point: §P<0·001.

Compliance was assessed by return of full and empty margarine pots and capsule packs.

[^{13}C]α-Linolenic acid tracer studies

Each subject participated in two tracer studies in order to determine the effect of altered n-3 FA intake upon their capacity to oxidise or convert [^{13}C]ALNA to longer-chain metabolites. The first was after 4 weeks of the run-in intervention (baseline tracer study) and the second after 8 weeks of randomisation to one of the interventions described earlier (post-intervention tracer study). Subjects were requested to avoid maize-based products for 3 d before metabolic studies (Jones et al. 1999) as these products are enriched in ^{13}C; this may result in raised background breath $^{13}CO_2$ enrichment.

Following an overnight fast, subjects arrived at the Hugh Sinclair Nutrition Unit at about 07.00 hours. Resting whole-body CO_2 excretion was determined using a GEM indirect calorimeter (PDZ-Europa, Crewe, Ches., UK) and baseline breath samples collected (Jones et al. 1999). A cannula was placed in a forearm vein and a baseline blood sample collected into a tube containing lithium heparin. The purpose of the study was to investigate the effects of alterations to the FA composition of the background diet on the capacity to convert [^{13}C]ALNA

Table 2. Fatty acid compositions of experimental spreads and capsules

Intervention group... Fatty acid‡	Fatty acid composition of spreads (g/100 g total fatty acids)*				Fatty acid composition of capsules (g/100 g total fatty acids)†	
	Run in	Control	High-ALNA	High-(EPA + DHA)	Control and high-ALNA	High (EPA + DHA)
12:0	3·33	2·29	2·58	1·69	0·20	0·20
14:0	1·00	1·02	1·07	1·19	0·70	5·50
16:0	13·6	13·6	13·2	12·6	34·90	16·1
18:0	3·76	3·71	3·76	4·19	4·60	3·10
22:0	0·50	0·49	0·2	0·60	ND	0·10
16:1n-7	0·10	0·07	0·05	0·57	ND	5·90
18:1n-9	23·4	23·6	23·7	23·0	35·3	11·6
20:1	0·25	0·24	0·14	0·30	ND	0·40
22:1	0·03	0·25	0·11	0·23	ND	2·10
24:1	ND	ND	ND	ND	ND	0·50
18:2n-6	52·5	51·4	12·9	50·8	ND	2·30
18:3n-3 (LNA)	0·96	0·93	40·7	0·30	ND	0·10
18:3n-6	ND	ND	ND	ND	ND	1·60
18:4n-3	ND	ND	ND	ND	ND	2·70
20:5n-3 (EPA)	ND	ND	ND	0·87	ND	11·0
22:6n-3 (DHA)	ND	ND	ND	1·33	ND	17·2

ALNA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ND, not detected.
* Spreads supplied by Unilever Health Institute (Vlaardingen, The Netherlands).
† Capsules supplied by Roche Vitamins Limited (Basel, Switzerland).
‡ 8:0, 10:0, trans-18:1 and 20:2 were present in these preparations at <0·5 g/100 g total fatty acids.

to longer-chain metabolites; thus, a standard test meal identical to that described by Burdge *et al.* (2002) was used in both the baseline and post-intervention tracer studies to allow direct comparison between measurements at these time points. [U-^{13}C]ALNA (700 mg) was consumed as a milk shake (160 ml) at about 08.00 hours together with a standardised breakfast, which provided a total energy intake from the test meal of 3995 kJ (protein 10·2, carbohydrate 49·1 and fat 40·7 % total energy). A second standardised meal with the same energy content was consumed 6 h later.

Breath specimens were collected at 1 h intervals over the first 10 h and then at 24 h. The number of blood samples (10 ml at each of five time points) was limited by the Ethical Committee to a total of 50 ml over 48 h as blood was also being collected for other measurements during the course of these tracer studies. Although the period of blood collection was shorter than we have reported previously (Burdge *et al.* 2002; Burdge & Wooton, 2002), this approach is directly comparable with that reported by Emken *et al.* (1994). In addition, the results of these analyses are directly comparable within the study, which permits the primary aim of assessment of the impact of the dietary interventions on ALNA metabolism to be determined within the same individuals. The timing of blood sampling was designed to reflect maximum [^{13}C]ALNA, [^{13}C]EPA and [^{13}C]DPA concentrations in plasma TAG and phosphatidylcholine (PC) as determined previously (Burdge *et al.* 2002) at 6, 10, 24 and 48 h. Since the number of time points was insufficient to use integration of area-under-the-curve to summarise the change in the concentration of labelled metabolites over time, results are presented as cumulative concentrations of individual ^{13}C-labelled FA over 48 h. The protocols for administration of [^{13}C]ALNA and specimen collection were identical for baseline and post-intervention tracer studies.

Analysis of total and ^{13}C-labelled fatty acid concentrations in blood lipids

Plasma lipids were isolated as described by Folch *et al.* (1957). Individual lipid classes were isolated by solid-phase extraction and FA methyl esters prepared as described by Burdge *et al.* (2000).

Enrichment with ^{13}C in individual FA was measured by GC–combustion isotope-ratio MS as described by Burdge & Wootton (2002) using tricosanoic acid methyl ester as the isotopic reference standard (1·135 atom %). Concentrations of individual labelled *n*-3 FA were calculated by integration of baseline-corrected peak areas on chromatograms derived from the total ion current and from fractional ^{13}C enrichment (Burdge & Wootton, 2002).

Estimation of ^{13}C-labelled fatty acid oxidation

Enrichment of ^{13}CO$_2$ in breath was determined in duplicate for each time point by continuous flow isotope-ratio MS using an automated breath C analyser (GSL; PDZ-Europa) interfaced to a 20/20 stable-isotope analyser

(PDZ-Europa) (Jones *et al.* 1999). ^{13}CO$_2$ enrichment and whole-body CO$_2$ excretion were used to estimate the proportion of administered FA which was oxidised and excreted on breath as ^{13}CO$_2$ over 24 h (Watkins *et al.* 1982; Jones *et al.* 1999).

Estimation of nutrient intakes by food-frequency questionnaire

Subjects completed a food-frequency questionnaire (Hartwell & Henry, 2001) before the start of the study and during the intervention period at approximately 5 months as described (Finnegan *et al.* 2003). Nutrient intakes were determined using FOODBASE (version 1.3; Institute of Brain Chemistry, London, UK).

Calculations and statistical analysis

Results are presented as mean values and standard deviations for total and cumulative ^{13}C-labelled FA concentrations. The relative cumulative concentrations of labelled FA in plasma were used to estimate apparent conversion of ALNA to longer-chain FA (Emken *et al.* 1994; Burdge *et al.* 2002; Burdge & Wootton, 2002). This was calculated by expressing the cumulative concentration of each ^{13}C-labelled FA as a percentage of the sum of the cumulative concentrations of labelled ALNA, EPA, DPA and DHA. Comparisons of individual FA concentrations for the whole study group were by Student's unpaired *t* test. The key question addressed in the present study is how measurements of plasma total and labelled FA concentrations, and of fractional oxidation, differed for each subject between baseline and after the intervention. This comparison was analysed by Student's paired *t* test. Statistical comparisons between groups either at baseline or after the intervention were by one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons. The analysis of specimens was conducted blind to the dietary intervention each subject received until calculation of results was complete. There was inadequate information in the literature (specifically the within-subject variability in ALNA conversion over time on a constant diet) to conduct a power analysis to determine sample size prior to conducting the present study. However, on the assumption that the within-subject variability in ALNA conversion to EPA is 10 % and that the amount of EPA formed from 10 000 nmol ALNA is 1000 (SD 100) nmol, then a sample size of *n* 4 or more would have >80 % power to detect a 30 % difference in conversion to EPA in a two-tailed test at $P < 0.05$.

Results

Subjects

There was no significant change in body weight within or between the individual intervention groups over the 8 weeks. Overall compliance to the margarine and capsules was >94 and >87 % respectively and was not significantly different among the groups. The mean daily intakes of *n*-3 FA, linoleic acid and total energy in the intervention

groups are summarised in Table 1. There were no significant differences in total energy, ALNA, EPA+DHA or linoleic acid intakes at baseline. After 5 weeks consuming the modified diets, total EPA+DHA intake was 6-fold greater in the high-(EPA+DHA) group, while ALNA and linoleic acid intakes were unchanged compared with baseline values. Consuming the high-ALNA diet increased ALNA intake 6-fold, while EPA+DHA and linoleic acid intakes were the same as baseline values.

Plasma lipid fatty acid compositions and labelled fatty acid cumulative concentrations at the baseline tracer study for the whole subject group

Total *n*-3 FA concentrations in plasma lipids from blood samples collected from fasted subjects on the day of the baseline tracer study after the 4-week run-in period are summarised for all fourteen subjects in Table 3. ALNA and DHA were the predominant *n*-3 FA in plasma TAG and non-esterified FA. In plasma PC, ALNA, EPA and

DPA were present at similar levels, while DHA concentration was 4- to 5-fold greater than other *n*-3 FA.

The cumulative concentrations of labelled FA for all subjects at the baseline tracer study for each lipid class are summarised in Table 3 and the pattern of changes in labelled ALNA, EPA, DPA and DHA concentrations in plasma PC are shown in Fig. 1. [13C]ALNA was the major labelled FA in all three lipid classes. The cumulative concentrations of labelled EPA and DHA were significantly greater in PC compared with TAG (2- and 3-fold respectively, $P<0.01$) and non-esterified FA (190- and 2-fold respectively, $P<0.01$). Cumulative concentration of [13C]DPA was similar in PC and TAG, but significantly lower (about 3-fold, $P<0.005$) in the non-esterified FA fraction. Previous studies have used the relative cumulative concentrations of each labelled *n*-3 FA in plasma total lipids to estimate the extent of apparent total conversion of ALNA to longer-chain metabolites (Emken *et al.* 1994; Burdge *et al.* 2002; Burdge & Wootton, 2002). Taking the same approach, the relative cumulative concentration of labelled FA in plasma total lipids in the current

Table 3. Plasma total and cumulative [13C]-labelled *n*-3 fatty acid concentrations at the baseline tracer study*
(Mean values and standard deviations for fourteen subjects)

	ALNA		EPA		DPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma total [13C]-labelled fatty acid concentrations (μmol/l)								
TAG	65	42	10	9	20	11	41	36
Non-esterified FA	5	3	1	1	2	1	6	1
PC	35	15	40	17	47	14	216	82
Cumulative [13C]-labelled FA concentrations (nmol/l over 48 h)								
TAG	50200	37796	653	557	624	622	8	7
Non-esterified FA	2100	973	6	2	8	4	2	1
PC	8500	4412	1093	625	182	152	20	16

ALNA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; TAG, triacylglycerol; FA, fatty acid; PC, phosphatidylcholine.
* For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312.
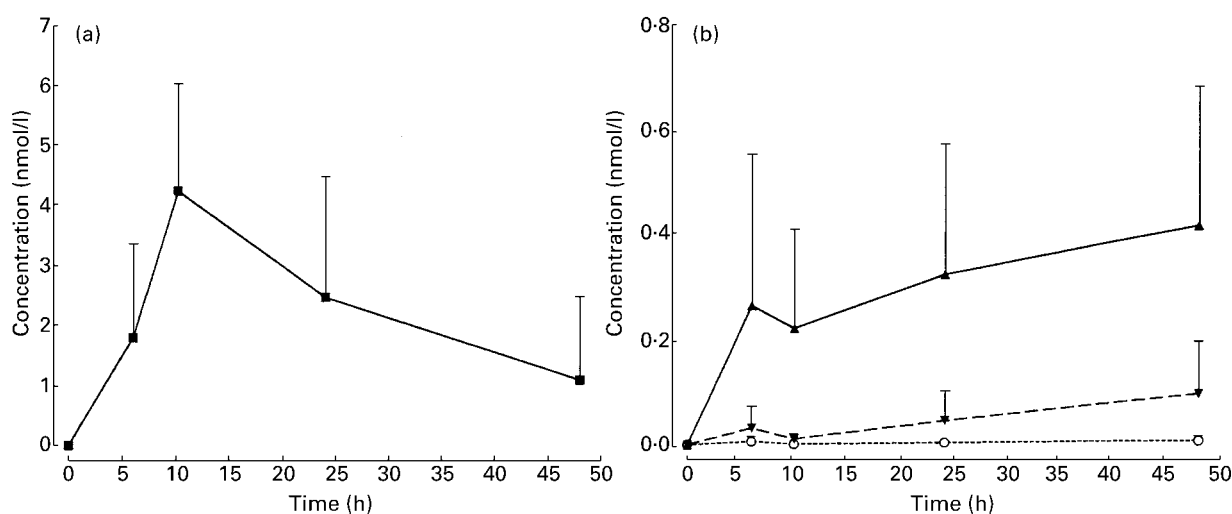
Fig. 1. Concentrations of [13C]-labelled *n*-3 fatty acids in plasma phosphatidylcholine following administration of [13C]-labelled α-linolenic acid (ALNA) at the baseline tracer study. (a), [13C]ALNA; (b), [13C]eicosapentaenoic acid (▲), [13C]docosapentaenoic acid (▼), [13C]docosahexaenoic acid (○). For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312. Values are means with standard deviations shown by vertical bars (*n* 14).

study were (%): ALNA 95·90, EPA 2·80, DPA 1·20 and DHA 0·04.

Effect of altered dietary n-3 fatty acid intake upon plasma fatty acid compositions

There were no significant differences between dietary groups in ALNA, EPA, DPA or DHA concentration or in cumulative concentrations of labelled FA in plasma TAG, non-esterified FA or PC at baseline (Table 4).

There was no significant effect of maintaining the control intervention for 8 weeks on TAG, non-esterified FA and PC concentrations of ALNA, EPA, DPA and DHA (Fig. 2). Increased consumption of ALNA significantly increased ($P < 0.05$) plasma TAG-EPA (4-fold) and -DPA (2-fold) concentrations, and EPA levels in non-esterified FA (6-fold) and PC (4-fold), at the post-intervention study compared with baseline. In addition, EPA and DPA concentrations were increased ($P < 0.05$) relative to both control and the high-(EPA+DHA) group in plasma TAG. However, EPA concentration at the post-intervention trial in plasma PC and non-esterified FA did not differ from the high-(EPA+DHA) group, but was greater than control ($P < 0.05$; Fig. 2). PC- and non-esterified FA-DPA concentration did not differ from control or high-(EPA+DHA) groups. Comparison of baseline and post-intervention studies within groups and between groups at the post-intervention study showed that increased ALNA intake did not alter ALNA or DHA concentrations significantly in plasma TAG, non-esterified FA or PC.

Increased consumption of EPA and DHA did not alter ALNA or DPA concentration in TAG, non-esterified FA or PC (Fig. 2). However, DHA concentration was increased significantly in the non-esterified FA fraction (2-fold) compared with baseline, and compared with the control and high-ALNA groups ($P < 0.05$). Both EPA (3-fold) and DHA (2-fold) were increased in plasma PC relative to baseline (Fig. 2). DHA concentration was significantly greater in the PC fraction compared with both the controls and high-ALNA group ($P < 0.05$), while EPA was only increased ($P < 0.05$) relative to the control group (Fig. 2). There was no effect of increased EPA+DHA intake on fasting plasma TAG n-3 FA concentrations.

Effect of dietary n-3 fatty acid intake upon the cumulative concentrations of labelled fatty acids in plasma lipids

Cumulative concentrations of ^{13}C-labelled FA at the baseline and post-intervention tracer studies are summarised in Table 4 and Fig. 3 respectively. There was no significant effect of the control, high-ALNA or high-(EPA+DHA) diets on [13C]ALNA incorporation into plasma TAG, non-esterified FA or PC. Neither the control nor high-ALNA diets altered the incorporation of labelled ALNA metabolites into plasma TAG, non-esterified FA and PC after the 8-week intervention period (Fig. 3). In contrast, increased consumption of EPA+DHA significantly reduced ($P < 0.05$) cumulative concentrations of [13C]EPA (2-fold) and [13C]DPA (4-fold) in plasma PC relative to baseline (Fig. 3). Cumulative labelled EPA and DPA concentrations
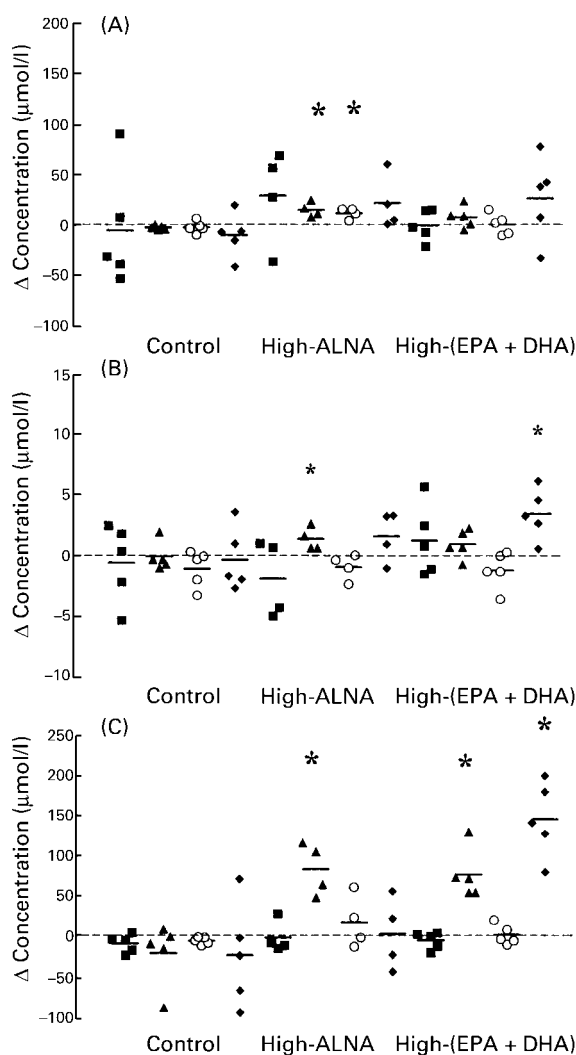
Table 4. Plasma *n*-3 fatty acid concentrations and cumulative concentrations of labelled fatty acids at the baseline tracer study*†

(Mean values and standard deviations)

Intervention group	Concentration (μmol/l)								Cumulative ^{13}C-FA concentration (nmol/l over 48 h)							
	ALNA		EPA		DPA		DHA		ALNA		EPA		DPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control (n 5)																
TAG	99	46	10	4	22	9	51	31	57400	42800	723	711	832	814	6	6
PC	40	12	42	17	50	7	260	89	5700	4100	602	647	77	85	11	9
Non-esterified FA	6	4	1	1	2	1	8	3	2489	139	2	1	9	5	2	1
High-ALNA (n 4)																
TAG	44	7	5	2	11	2	14	7	50500	28142	482	364	456	476	10	10
PC	30	17	44	16	48	11	207	41	10200	3599	1215	373	196	149	19	15
Non-esterified FA	7	3	1	1	2	1	5	2	2158	350	13	15	6	4	3	2
High-(EPA+DHA) (n 5)																
TAG	49	38	14	12	24	13	53	45	42700	42471	732	545	480	502	7	3
PC	31	16	34	20	42	20	180	91	10000	4262	1582	301	277	140	32	23
Non-esterified FA	7	3	1	1	3	1	5	2	1545	476	2	2	8	4	3	2

FA, fatty acid; ALNA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; TAG, triacylglycerol; PC, phosphatidylcholine.
* For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312.
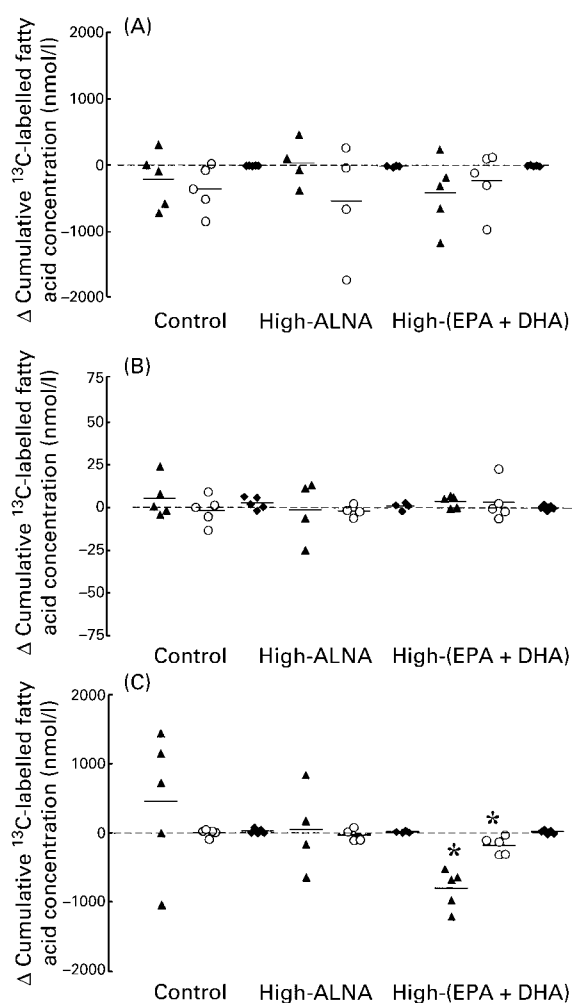† There were no significant differences (assessed by one-way ANOVA) between dietary groups within each lipid class at baseline.

Fig. 2. Difference in plasma *n*-3 polyunsaturated fatty acid concentrations between tracer studies (post-intervention − baseline). (A), triacylglycerol; (B), non-esterified fatty acids; (C), phosphatidylcholine. Values are results for individual subjects. —, Mean value. For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312. ■, α-linolenic acid (ALNA); ▲, eicosapentaenoic acid (EPA); ◆, docosapentaenoic acid; ○, docosahexaenoic acid (DHA). Mean values were significantly different between baseline and post-intervention: *$P < 0.05$. The results of comparisons between dietary groups are given on pp. 315–317.

**Fig. 3.** Difference in cumulative ^{13}C-labelled *n*-3 polyunsaturated fatty acid concentrations in plasma between tracer studies (post-intervention − baseline). (A), triacylglycerol; (B), non-esterified fatty acids; (C), phosphatidylcholine. Values are results for individual subjects. —, Mean value. For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312. ▲, eicosapentaenoic acid (EPA); ◆, docosapentaenoic acid; ○, docosahexaenoic acid (DHA). Mean values were significantly different between baseline and post-intervention: *$P < 0.05$. The results of comparisons between dietary groups are given on pp. 315–317. Cumulative [^{13}C]α-linolenic acid concentration did not differ between tracer studies or between groups (results not shown). ALNA, α-linolenic acid.

in plasma PC were also significantly ($P < 0.05$) lower than the control and high-ALNA groups at the post-intervention study (Fig. 4). There was no significant effect of increasing EPA+DHA intake on the cumulative concentrations of labelled ALNA or DHA in plasma PC or on cumulative concentrations of labelled EPA, DPA or DHA in plasma TAG or non-esterified FA (Fig. 3).

The ^{13}C-enrichment of ALNA in plasma PC at 6 h was used to assess whether there were significant differences in isotopic dilution with unlabelled ALNA between the tracer studies. There was no significant difference between tracer studies in ^{13}C-enrichment of ALNA in plasma PC in the control (baseline 3·1 (SD 1·2), post-intervention 2·8 (SD 1·3) atom % excess), high-ALNA (baseline 2·9 (SD 1·2), post-intervention 3·0 (SD 1·3) atom % excess)

and high-(EPA+DHA) (baseline 3·9 (SD 1·2), post-intervention 3·2 (SD 0·9) atom % excess).

Effect of dietary n-3 fatty acid intake upon recovery of α-[^{13}C]linolenic acid as ^{13}CO$_2$ on breath

The proportion of the administered dose of [^{13}C]ALNA recovered on breath as ^{13}CO$_2$ over 24 h was calculated from enrichment above natural ^{13}C abundance and whole-body CO$_2$ excretion (Watkins *et al.* 1982; Jones *et al.* 1999). In the present study no attempt was made to correct these values for trapping of ^{13}CO$_2$ in body bicarbonate pools. The pattern of change in ^{13}CO$_2$ excretion on breath at baseline is shown in Fig. 4. At the baseline
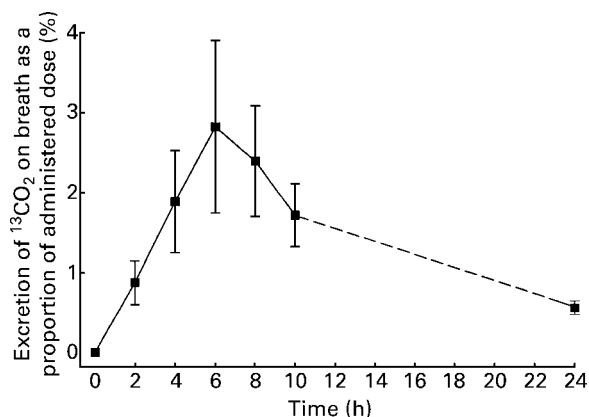
Fig. 4. Excretion of $^{13}CO_2$ in breath expressed as a proportion of the administered dose of α-[^{13}C]linolenic acid. For details of subjects, interventions and procedures, see Tables 1 and 2 and p. 312. Values are means with standard deviations shown by vertical bars (*n* 14).

metabolic trial the mean fractional oxidation for all fourteen subjects was 33·8 (SD 1·9)% (27 3 (SD 13) mg) over 24 h. There was no significant difference between dietary groups at the baseline tracer study: control 33·1 (SD 4·7)% (231 (SD 33) mg), high-ALNA 36·1 (SD 2·0)% (253 (SD 14) mg), high-(EPA+DHA) 32·3 (SD 2·6)% (226 (SD 18) mg). Maintaining the control diet for 8 weeks did not change fractional oxidation of [^{13}C]ALNA over 24 h (38·9 (SD 2·2)% (273 (SD 22) mg)) significantly. The proportion of [^{13}C]ALNA recovered as $^{13}CO_2$ on breath at the post-intervention trial was not altered significantly by either increasing ALNA intake (34·3 (SD 4·4)% (239 (SD 10) mg)) or EPA+DHA consumption (38·3 (SD 3·2)% (268 (SD 232) mg)) relative to baseline or between dietary groups.

Discussion

The results of the present study showed that these men were capable of limited conversion of ALNA to longer-chain FA, including DHA. The results from subjects who maintained the control intervention throughout the study showed that there was no evidence of significant temporal variation in the extent of ALNA conversion over 8 weeks. Increasing ALNA intake increased plasma PC-EPA concentration, while increased EPA+DHA consumption raised plasma PC-EPA and -DHA concentrations. Increased ALNA or EPA+DHA produced differing effects on conversion of [^{13}C]ALNA to longer-chain FA. There was no significant change in the cumulative concentration of labelled *n*-3 FA in plasma following increased consumption of ALNA for 8 weeks. In contrast, increased EPA+DHA consumption resulted in lower cumulative concentrations of labelled EPA and DPA. DHA synthesis and the extent of partitioning of [^{13}C]ALNA towards β-oxidation were essentially unaffected by time over 8 weeks or by any of the dietary manipulations.

There were marked differences between subjects in both plasma FA concentrations and cumulative concentrations of labelled FA, which was emphasised by the small number of subjects studied. The high cost and technical

demands of stable-isotope tracer analysis limit the number of subjects studied: this is a characteristic factor of all studies reported in the literature so far (Emken *et al.* 1994, 1999, 2002; Salem *et al.* 1999; Vermunt *et al.* 2000). The degree of variation between individuals in the concentrations of labelled FA reported here is consistent with that described by others. For example, Salem *et al.* (1999) showed, in individuals who consumed [^{2}H]ALNA, peak labelled EPA and DHA concentrations of approximately 92 and 72% respectively ((SD/mean) ×100) in thirteen subjects. In addition, others showed 25% variation in labelled EPA following administration of [^{13}C]ALNA (Vermunt *et al.* 2000). Whether this variation reflects differences in diet or innate physiological and biochemical factors has yet to be determined. However, such variation may represent an important consideration in the design of nutritional interventions to modify *n*-3 FA status.

Maintaining the control diet showed that the composition of plasma lipids was essentially stable over the 8-week period. In fasting subjects plasma PC and TAG compositions are the net product of hepatic lipid synthesis, export as VLDL and turnover within the plasma compartment. Fasting plasma non-esterified FA composition reflects primarily that of FA mobilised from adipose tissue. Thus, these results suggest that these processes are regulated closely and are stable over at least short periods of time. Increased ALNA consumption did not produce a significant increase in ALNA concentration in any of the plasma lipid classes analysed, although there was a trend towards an increase in ALNA in the TAG fraction. The absence of a significant change probably reflects the variation between subjects and the small sample size (for example, see Fig. 2). However, increased ALNA intake was associated with modest increases in EPA and DPA concentrations, but there was no significant effect on DHA concentration. This is in agreement with other reports of the effect of increased ALNA intake on plasma lipid composition (Sanders & Rosahani, 1983; Kwon *et al.* 1991; Chan *et al.* 1993; Freese *et al.* 1994; Allman *et al.* 1995; Cunnane *et al.* 1995; Mantzioris *et al.* 1995; Li *et al.* 1999; Finnegan *et al.* 2001, 2003). The subjects involved in the present study formed part of a larger, longer-term (6 months) study. Findings for plasma lipid FA compositions for the whole group and for the 6-month period demonstrated significant elevation in plasma phospholipid ALNA concentration, and confirm and reinforce the observation made here that ALNA increases EPA but not DHA concentrations in plasma lipids (Finnegan *et al.* 2003). These compositional results are consistent with the view that conversion of ALNA to EPA and DPA is limited, while DHA synthesis is severely constrained (Gerster, 1998). As expected, increased EPA and DHA intakes increased only the levels of these FA in plasma lipids.

At the baseline tracer study, ^{13}C enrichment was detected in ALNA, EPA, DPA and DHA in plasma TAG, non-esterified FA and PC following the ingestion of [^{13}C]ALNA. Labelled ALNA was the predominant enriched FA in all three lipid classes, while [^{13}C]DHA exhibited the lowest cumulative concentration. In addition, the TAG fraction

showed the greatest cumulative concentration of [13C]ALNA, while plasma PC showed the greatest cumulative concentration of labelled DHA. This differential distribution broadly reflects that of total ALNA and DHA between plasma lipid classes. The experimental approach used in this and other studies is likely to produce an underestimate of total ALNA inter-conversion, since only FA mobilised from the liver and appearing in the plasma pool can be measured. This approach does not permit estimation of losses of ALNA metabolites due to partitioning towards oxidation and storage, or the contribution of selective incorporation of FA into lipids secreted by the liver. In addition, the concentrations of labelled FA in plasma reflect the net product of newly synthesised FA entering the circulation and FA already present, rather than simply the mobilisation of newly synthesised FA. However, in the absence of techniques for measuring ALNA conversion directly, the experimental protocol used in the current study and by others represents the only practical approach to assessment of capacity for ALNA conversion in man *in vivo*.

Since there is a lack of uniformity between studies of FA conversion in the presentation of results, comparison of the present results with previous reports is difficult. The relative cumulative concentration of individual labelled FA has been used previously to estimate the extent to which ALNA was converted to longer-chain metabolites (Emken *et al.* 1994; Burdge *et al.* 2002; Burdge & Wootton, 2002). The relative cumulative concentrations of labelled EPA, DPA and DHA in total plasma lipids in the present study were lower than reported previously over 48 h (EPA 8·0, DPA 4·2, DHA 4·0 %; Emken *et al.* 1994). Calculation of the [13C]DHA:[13C]DPA ratio (sum of cumulative concentrations in plasma TAG, non-esterified FA and PC), which reflects the relative synthesis of DPA and DHA, permits some comparison with studies in which results are not presented as relative cumulative concentrations. In the present study, the [13C]DHA:[13C]DPA ratio (0·04) for all fourteen subjects at the baseline tracer study was comparable with that reported by Salem *et al.* (1999) (0·02) and Vermunt *et al.* (2000) (0·06). Despite differences in experimental design and the form in which results were expressed, these present results are consistent with previous studies (Emken *et al.* 1994; Salem *et al.* 1999; Vermunt *et al.* 2000; Pawlosky *et al.* 2001) in demonstrating that conversion of ALNA to EPA and DPA is limited, and that DHA synthesis is marginal. Overall, comparisons between the present results and those reported previously in younger individuals suggest that there was no marked effect of increasing age on capacity for ALNA conversion. This suggests that, at least in men, conversion of ALNA to longer-chain metabolites remains low, but does not decline substantially throughout adult life.

There were no significant differences between baseline and post-intervention tracer studies in the mean cumulative concentrations of 13C-labelled *n*-3 FA in the group maintained on the control intervention. This suggests that capacity for ALNA conversion was relatively stable for the group over the 8-week intervention period. However, within the control group some subjects showed opposing trends between the tracer studies, for example in cumulative [13C]EPA concentration (Fig. 3). Such individual variation

in polyunsaturated FA metabolism may be an important determinant of the efficacy of ALNA supplementation in altering circulating FA concentrations. Increased ALNA intake resulted in higher EPA and/or DPA concentrations in plasma lipids, suggesting increased conversion of substrate to these longer-chain products. However, there was no significant effect of this intervention upon the cumulative concentrations of labelled FA. There are two possible explanations for this discrepancy between the effects of increased ALNA intake on EPA and DPA concentrations in plasma, but lack of an effect on cumulative concentrations of labelled EPA and DPA measured following a single bolus of [13C]ALNA. One is that increased ALNA intake led to increased conversion to EPA and DPA and thereby increased EPA and DPA concentrations in plasma at an earlier stage in the dietary intervention. Once tissue concentrations reached a threshold, rates of conversion returned to baseline to maintain steady-state concentrations of these FA. The other possible explanation is that increased intake of unlabelled ALNA would have diluted the labelled FA, leading to a net decrease in rate of the entry of [13C]ALNA into the desaturation–elongation pathway. Thus, in order for similar cumulative [13C]EPA and [13C]DPA concentrations to have been measured in plasma at baseline and at the end of the supplementation period, the overall rate of synthesis must have been greater during the period of increased ALNA consumption. However, measurement of the enrichment of ALNA in plasma PC at 6 h, the earliest sampling time and thus the most reflective of hepatic FA pools destined for mobilisation, showed no significant difference between tracer studies before and after the dietary intervention or between dietary groups. This suggests that dilution of [13C]ALNA with unlabelled ALNA is unlikely to account for these results and that the former suggestion is more likely to be true. The lack of an effect of increased ALNA consumption on conversion of labelled ALNA is in contrast with the report of Vermunt *et al.* (2000), who showed increased ALNA consumption to inhibit [13C]ALNA conversion. However, the cause of this apparent discrepancy is not clear, since the precise mechanism by which increased ALNA intake inhibited [13C]ALNA conversion in not known.

Increased EPA+DHA consumption was associated with a decrease in the cumulative concentrations of labelled EPA and DPA, but not DHA. This may reflect feedback inhibition of ALNA conversion to EPA and DPA due to accumulation of EPA and DHA, the major products of ALNA desaturation and elongation (Emken *et al.* 1999; Vermunt *et al.* 2000).

Conversion of [13C]ALNA to DHA was extremely low and appeared to be largely unaffected by any of the dietary interventions. Although increased ALNA intake may have been associated with increased conversion to EPA and DPA, there was no change in cumulative [13C]DHA concentration or total DHA concentration. This is confirmed by the observation that there was no significant change in plasma phospholipid DHA concentration even after 6-months supplementation with 10 g ALNA/d (Finnegan *et al.* 2003). DHA synthesis involves translocation between the endoplasmic reticulum and limited peroxisomal β-oxidation, which have been suggested to be potential

metabolic control points for the pathway (Sprecher, 2000). This may permit regulation of DHA synthesis independently from that of EPA and DPA. Furthermore, while synthesis of EPA and DPA was reduced by increased EPA+DHA intake, again cumulative labelled DHA concentration was not different from baseline. One possible explanation is that while DPA synthesis may have been down-regulated, there may have been sufficient DPA to sustain DHA synthesis. Previous studies have reported decreased DHA synthesis following increased DHA or EPA+DHA consumption (Emken et al. 1999; Vermunt et al. 2000). However, this may reflect differences in EPA+DHA intake between the present study and those reported previously. The study by Vermunt et al. (2000) used a similar total EPA+DHA intake (1·5 g/d) to the present report, but the EPA:DHA ratio was about 2·0, while that reported here was about 0·6. Emken et al. (1999) used purified DHA alone to provide a substantially greater dose (6·5 g/d) than used in the present study, and this may be assumed to potentially exert a greater effect on DHA synthesis. In addition, the cross-sectional study design used by Emken et al. (1999) and Vermunt et al. (2000) compared with the longitudinal design used in the present study may have contributed to differences between reports on the effects of increased EPA+DHA consumption on DHA synthesis.

The proportion of [^{13}C]ALNA recovered as $^{13}CO_2$ on breath at the baseline tracer study was about 34 % over 24 h. However, estimates of retention of $^{13}CO_2$ within body bicarbonate pools suggest that the actual extent of partitioning of labelled ALNA towards oxidation may have been up to 30 % greater than the measured value (Irving et al. 1983). In addition, absence of collection of breath samples overnight may have reduced the precision of the estimate of the fractional oxidation of ALNA, although it is unclear whether this would have resulted in an under- or over-estimate. The proportion of labelled FA excreted as $^{13}CO_2$ on breath in the present study was of a similar order of magnitude to that reported previously (Vermunt et al. 2000; Bretillon et al. 2001; Burdge et al. 2002). The proportion of [^{13}C]ALNA recovered on breath as $^{13}CO_2$ was similar to that reported for [1-^{13}C]palmitic acid under comparable experimental conditions (Jones et al. 1998, 1999; Bennoson et al. 1999), which indicates that there was no preferential use or sparing of ALNA as a metabolic fuel. There was no significant effect of increasing either ALNA or EPA+DHA intakes upon recovery of $^{13}CO_2$ on breath. Comparison of $^{13}CO_2$ excretion at baseline and following the three dietary interventions suggests that neither increased ALNA or EPA+DHA substantially altered the partitioning of ALNA towards β-oxidation. Since increasing ALNA intake 10-fold did not increase [^{13}C]ALNA oxidation, plasma ALNA concentration, or conversion of ALNA to longer-chain polyunsaturated FA, the additional ALNA must have been partitioned into storage pools within the body. There was no evidence that increased EPA+DHA intake altered partitioning of ALNA towards β-oxidation.

The results of the present study indicate that while increased intakes of EPA and DHA reduced ALNA conversion, probably by product inhibition of Δ6- and/or

Δ5-desaturase, increased consumption of ALNA did not alter EPA and DHA synthesis. In contrast, DHA synthesis appears to be regulated tightly and apparently independently from other ALNA metabolites. One potential role of such metabolic control may be to ensure adequate availability of DHA within the body irrespective of changes in dietary FA intake. Overall, these results suggest that increased ALNA consumption may be a useful means for increasing synthesis of EPA and DPA, but not DHA. This may be of particular importance to individuals in the age range studied here who are at increased risk of cardiovascular disease, since EPA has potential beneficial effects independent of DHA (Babcock et al. 2000; Mori et al. 2000; McLennan, 2001; Shimokawa, 2001).

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