

Low-*n*-6 and low-*n*-6 plus high-*n*-3 diets for use in clinical research

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(Submitted 20 April 2012 – Final revision received 16 October 2012 – Accepted 17 October 2012 – First published online 18 January 2013)

Abstract

Few trials have evaluated the metabolic effects and health outcomes of lowering dietary *n*-6 PUFA. The objectives of the present paper were (1) to report the methods employed to lower dietary *n*-6 PUFA, while either increasing or maintaining *n*-3 PUFA intake and (2) to validate our methods with 24 h recalls and erythrocyte fatty acid analyses. A total of sixty-seven subjects were randomised to either (1) an average-*n*-3 PUFA, low-*n*-6 PUFA (L6) intervention designed to lower linoleic acid (LA; $\leq 2.5\%$ of energy (en%)) and arachidonic acid (≤ 60 mg/d), while maintaining an average US intake of *n*-3 PUFA or (2) a high-*n*-3 PUFA, low-*n*-6 PUFA (H3-L6) intervention designed to lower *n*-6 LA, while increasing the *n*-3 PUFA α -linolenic acid (ALA; ≥ 1.5 en%) and EPA + DHA (≥ 1000 mg/d). Pre- and intra-intervention nutrient intakes were estimated with six 24 h dietary recalls per subject. Both groups achieved the targeted reductions in dietary LA to ≤ 2.5 en% (median LA 2.45 (2.1, 3.1); $P < 0.001$). Intakes of *n*-3 PUFA did not change for the L6 group. Target increases in *n*-3 ALA (median 1.6 en%, (1.3, 2.0), $P < 0.001$) and EPA + DHA (1482 mg, (374, 2558), $P < 0.001$) were achieved in the H3-L6 group. Dietary changes were validated by corresponding changes in erythrocyte *n*-6 and *n*-3 fatty acid composition. Dietary LA can be lowered to ≤ 2.5 en%, with or without concurrent increases in dietary *n*-3 PUFA, in an outpatient clinical trial setting using this integrated diet method.

Key words: *n*-6 Fatty acids: *n*-3 Fatty acids: Polyunsaturated fatty acids: Dietary interventions: Headache

Modern per capita US *n*-6 linoleic acid (LA) intake of 6.3–6.7% of energy (en%)⁽¹⁾ is substantially higher than available data estimating historical US (2.2 en%)⁽²⁾ and evolutionary (2.3–3.6 en%) LA consumption⁽³⁾. Since the early 1960s, dietary advice to substitute vegetable oils for animal fats contributed to major changes in the US food supply, including a reduction in SFA⁽¹⁾ and a 3-fold increase in LA intake⁽²⁾. Data on the metabolic and health effects of these marked dietary

changes are limited⁽⁴⁾. Dietary *n*-6 and *n*-3 PUFA are known to alter the composition and metabolic functions of various tissues in both animal models^(5–8) and human subjects^(4,9,10). Several, but not all, controlled trials comparing *n*-3 PUFA supplements *v.* placebo have demonstrated substantial health benefits^(11–15).

Since *n*-6 and *n*-3 fatty acids compete to some extent for enzymatic conversion, tissue incorporation and metabolism to bioactive derivatives, lowering dietary LA could potentially

Abbreviations: AA, arachidonic acid; en%, percentage of energy; H3-L6, high-*n*-3, low-*n*-6 diet; IQR, interquartile range; L6, average-*n*-3, low-*n*-6 diet; LA, linoleic acid; NDSR, Nutritional Data System for Research.

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augment the benefits of *n*-3 fatty acids or have substantial independent health benefits^(15–17). To our knowledge, no human trial has evaluated the long-term effects of lowering dietary LA from modern intakes to amounts consistent with historical US or evolutionary intakes for the prevention or management of any medical condition.

Challenges for evaluating the effects of dietary LA lowering in Western populations include: (1) the ubiquity of LA in the food supply, with LA-rich oils added to most packaged food items; (2) wide variability in the LA content of many similar food items; and (3) the abundance of LA in adipose tissue stores of Western populations. Because LA has a slow turnover rate (half-life 1–2 years) in adipose tissue^(18,19), dietary changes may need to be maintained for relatively long periods to achieve maximal alterations in tissue composition and function.

With these unique challenges in mind, we developed a novel, integrated diet method designed to achieve marked reductions in dietary LA in an outpatient setting. Subjects with chronic daily headaches were randomised to one of two low-*n*-6 study diets: (1) an average-*n*-3, low-*n*-6 (L6) diet and (2) a high-*n*-3, low-*n*-6 (H3-L6) diet. The interventions combined four key elements: (1) provision of both unprepared and specially formulated prepared foods accounting for two-thirds of total energy intake; (2) intensive dietitian-administered counselling, with targeted and individually tailored aspects; (3) continuous self-monitoring; and (4) access to an intervention-specific website to complement and reinforce dietitian advice.

This novel dietary method was developed to achieve: (1) a reduction in *n*-6 LA intake to ≤ 2.5 en% in both intervention groups; and (2) an increase in *n*-3 EPA + DHA to ≥ 1000 mg/d in the H3-L6 group, with no change in the L6 group. These goals were evaluated by administering six 24 h dietary recalls per subject. Erythrocyte fatty acids were analysed to provide further evidence of adherence. Complete metabolic and clinical headache outcomes will be presented in a separate paper.

Experimental methods

Participants

Individuals who were at least 18 years old and who satisfied the 2004 International Classification of Headache Disorders criteria for chronic daily headaches⁽²⁰⁾ were invited to participate. Patients with chronic daily headaches, defined as at least fifteen headache days per month and 4 h/d⁽²¹⁾, were recruited from the following sources: (1) specialty headache clinics; (2) broadcast email to the University of North Carolina (UNC) community; and (3) brochures placed in UNC medical clinics. This population with chronic and frequent pain was selected in order to evaluate the hypothesis that targeted alterations in dietary fatty acids can reduce the frequency and severity of pain outcomes. Pregnant women and those regularly consuming fatty acid supplements were excluded. The present study was conducted according to the Declaration of Helsinki guidelines, with all procedures involving human subjects

approved by the UNC-Chapel Hill Institutional Review Board. All participants provided written informed consent. This trial is registered under ClinicalTrials.gov (NCT01157208).

Development of study diets

Two research diets were developed for the 12-week, parallel-group randomised dietary intervention. The L6 diet was designed to reduce LA and arachidonic acid (AA) to ≤ 2.5 en% and 60 mg/d, respectively, while providing average US intakes of ALA and EPA + DHA. The H3-L6 diet was designed to reduce LA to ≤ 2.5 en%, while increasing ALA and EPA + DHA. Both diets were designed to provide typical US amounts of total protein, total carbohydrate and total fat.

The average-*n*-3, low-*n*-6 diet

Lowering *n*-6 PUFA. LA (18:2*n*-6) is the predominant PUFA in US/Western diets⁽¹⁾. High-LA oils (soyabean, maize, cottonseed, safflower and sunflower) are the predominant fat sources in most US salad dressings, margarines, mayonnaises and cooking oils. LA-rich oils are also added to most packaged food items, including breads, cereals, soups and tomato sauce, as well as most snack foods such as cookies, chips (crisps) and crackers (Supplementary Appendix 1, available online).

To achieve the intervention-specific LA intake targets, study foods were carefully selected to replace those containing substantial quantities of LA (Supplementary Appendix 1, available online). The United States Department of Agriculture National Nutrient Database for Standard Reference, Release 21, was used to screen high-LA and low-LA foods for potential inclusion and exclusion from research diets. Next, to verify low LA content, the fatty acid content of multiple brands of low-LA foods was analysed by GC, as previously described⁽²²⁾. The lowest-LA foods were selected for inclusion in both diets. To facilitate dietary LA lowering, participants were provided with, and instructed to exclusively use, study-provided low-LA oils and fat sources (Table 1), including coconut oil, macadamia nut oil, low-LA olive oil, butter, fat-free mayonnaise and macadamia-vinaigrette salad dressing. Because most US packaged food items contain substantial amounts of added LA, participants also received a variety of low-LA substitute foods, including crackers, tortillas, breads and popcorn. Research foods were procured and prepared by the UNC Nutrition Research and Metabolism Core of the Clinical and Translational Research Center.

Lowering dietary arachidonic acid. Foods with high AA (20:4*n*-6) content, according to the United States Department of Agriculture National Nutrient Database for Standard Reference, Release 21, were first identified as key items to eliminate from the research diets (Supplementary Appendix 1, available online). Fatty acid analysis was then performed on several animal protein sources in order to identify study foods containing the lowest amounts of both LA and AA (Table 1). Participants were instructed to restrict consumption of egg yolks, meat, most poultry and certain seafood. Adequate protein intake was achieved with beans, very lean (low *n*-3) seafood, turkey, egg-whites and dairy products. Examples of

study-provided protein sources included canned beans, prepared vegetarian chili, frozen low-fat seafood, cheeses and low-fat turkey (Table 1).

Average n-3 PUFA. The average US intake of ALA (18:3n-3) is about 0.6 en%⁽¹⁾, mostly from soyabean and rapeseed oils. Because these vegetable oil sources of ALA were restricted, a small amount of ground flaxseed (concentrated source of ALA) was added to L6 diet foods to maintain the average US ALA intake. Participants had the option of consuming specially formulated muffins, granola or 3 g of ground flaxseed added to foods of their choice. To achieve the average US EPA + DHA intake of 0.11 g/d, participants received small amounts of seafood with the lowest amounts of EPA + DHA and AA (Table 1).

The high-n-3, low-n-6 diet

Lowering dietary linoleic acid. H3-L6 dieters were instructed to replace all high-LA foods in their diet with the same study-provided oils, fats and other low-LA foods used in the L6 diet (Table 1).

Increasing dietary n-3 PUFA. H3-L6 subjects were instructed to consume approximately 1.5 en% as ALA (Table 2), mostly from ground flaxseed. Options included 14 g of ground flaxseed each day for addition to foods of their choice, or specially prepared granola, muffins, granola bars and/or bean dip containing flaxseed. To meet EPA + DHA intake targets, H3-L6 dieters were counselled to consume 113–230 g of fatty fish or shellfish per d in place of other protein sources. Fatty acid analyses

Table 1. Linoleic acid (LA), α -linolenic acid (ALA), arachidonic acid (AA), EPA and DHA present in 100 g portions of study-provided foods*

	Analysis	LA (g)	ALA (g)	AA (mg)	EPA (mg)	DHA (mg)	Diet group
Plant foods							
Coconut oil, unrefined	SNN	0.97	0.0	NA	NA	NA	Both
Macadamia nut oil	SNN	3.34	0.34	NA	NA	NA	Both
Extra virgin olive oil (Trader Joe's brand)	SNN	6.21	0.65	NA	NA	NA	Both
Macadamia nut oil vinaigrette†	FP	0.38	0.04	NA	NA	NA	Both
Italian vinaigrette (Kraft)	SNN	2.72	0.89	NA	NA	NA	Both
Fat-free mayonnaise	SNN	1.05	0.15	NA	NA	NA	Both
Flaxseed, ground	SNN	5.15	15.42	NA	NA	NA	Both
Popcorn, no added fat	SNN	3.19	0.06	NA	NA	NA	Both
Tortilla (Tunaro's brand)	SNN	0.84	0.16	NA	NA	NA	Both
Crackers, low fat	USDA	0.65	0.04	NA	NA	NA	Both
Whole-wheat bread (Ninth Street Bakery)	SNN	0.6	0.04	NA	NA	NA	Both
Granola†	FP	0.54	1.18	NA	NA	NA	L6‡
Granola with flaxseed†	FP	0.97	2.12	NA	NA	NA	H3-L6§
Bean dip†	FP	0.84	0.05	NA	NA	NA	L6
Bean dip with flaxseed†	FP	0.55	1.1	NA	NA	NA	H3-L6
Muffins†	FP	0.4	0.8	NA	NA	NA	L6
Muffins with flaxseed†	FP	0.7	1.58	NA	NA	NA	H3-L6
Flaxseed granola bars (1)†	FP	0.8	1.0	NA	NA	NA	H3-L6
Mozzarella cheese string	USDA	0.34	0.14	NA	NA	NA	Both
Blueberries, frozen	USDA	0.17	0.11	NA	NA	NA	Both
Vegetarian chili†	FP	0.14	0.05	NA	NA	NA	Both
Garbanzo beans	USDA	0.49	0.02	NA	NA	NA	Both
Black beans	USDA	0.13	0.11	NA	NA	NA	Both
Kidney beans	USDA	0.11	0.08	NA	NA	NA	Both
Mixed vegetables, frozen	USDA	0.05	0.02	NA	NA	NA	Both
Mixed fruit, frozen	USDA	0.02	0.01	NA	NA	NA	Both
Animal foods							
Butter, salted	SNN	2.98	0.38	0.18	0.04	0.04	Both
Yogurt, low fat	USDA	0.02	0.01	NA	NA	NA	Both
Cod, filet	SNN	0.01	0.0	0.01	0.05	0.12	L6
Tuna, low-fat chunk light	SNN	0.01	0.0	0.03	0.02	0.18	L6
Grouper, filet	SNN	0.0	0.0	0.02	0.01	0.13	L6
Shrimp	SNN	0.05	0.0	0.01	0.05	0.07	Both
Turkey, low fat	SNN	0.15	0.02	0.05	0.0	0.01	L6
Wild salmon, filet	SNN	0.02	0.01	0.01	0.14	0.43	H3-L6
Wild albacore tuna, canned (Vital choice brand)	SNN	0.21	0.13	0.13	1.0	3.15	H3-L6
Wild salmon, canned (Vital choice brand)	SNN	0.09	0.06	0.03	0.49	0.81	H3-L6
Trout, filet	SNN	0.07	0.01	0.02	0.09	0.34	H3-L6
Swordfish, filet	SNN	0.01	0.0	0.04	0.02	0.25	H3-L6
Tuna, steaks	SNN	0.01	0.0	0.04	0.03	0.24	H3-L6

SNN, Section on Nutritional Neurosciences, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism (NIAAA), National Institutes of Health; NA, not applicable; FP, ESHA Food Processor 10.6.0.0 (analyses labelled FP used a combination of United States Department of Agriculture (USDA) and NIAAA data); USDA, USDA Nutrient Database for Standard Reference, version 21.

* USDA SR21 nutrient values were used for diet calculations for foods with low absolute amounts and variability of n-6 LA. ESHA Food Processor 10.6.0.0 software was used to analyse study-provided recipes. To account for differences between study-provided foods and software values, values from direct fatty acid analysis of study foods were added to the database.

† Indicates specially formulated foods supplied via the metabolic kitchen.

‡ L6: average-n-3, low-n-6 diet.

§ H3-L6: high-n-3, low-n-6 diet.

Table 2. Demographics

(Number of subjects and percentages; mean values and standard deviations)

	Total sample (n 67)		L6 diet (n 34)		H3-L6 diet (n 33)	
	n	%	n	%	n	%
Sex						
Male	9	13.4	4	11.6	5	15.2
Female	58	84.6	30	88.2	25	80.6
Race/ethnicity						
White	58	86.6	30	88.1	28	84.8
African-American	7	10.5	3	8.8	4	12.1
American Indian	2	3.0	1	3.0	1	3.2
Marital status						
Married	38	56.7	19	55.9	19	57.5
Partnered	9	13.4	5	14.7	4	12.1
Single	13	19.4	6	17.6	7	21.2
Widowed, divorced	7	10.4	4	11.8	3	9.1
Education						
High school	4	6.1	2	6.2	2	6.1
Some college	16	24.2	6	18.7	10	30.3
Bachelor's degree	19	29.2	11	34.4	8	24.2
Master's or higher	26	40.0	13	39.4	13	40.6
Employment						
Employed	44	66.7	21	63.6	23	69.7
Student	5	7.6	2	6.1	3	9.1
Retired/caretaker	6	9.1	3	9.1	3	9.1
Disabled/unemployed	11	16.7	7	20.0	4	12.1
Age (years)						
Range: 18.7–62.4						
Mean		41.6		42.5		41.0
SD		11.5		11.0		12.3

L6, average-n-3, low-n-6 diet; H3-L6, high-n-3, low-n-6 diet.

were performed on numerous types and brands of seafood to ensure selection of those with the highest EPA + DHA and low amounts of LA and AA (Table 1). The study-provided foods included frozen wild salmon fillets, trout fillets, tuna steaks, canned high-n-3 albacore tuna, canned high-n-3 wild salmon and sardines. The small amount of AA present in study-provided seafood resulted in daily AA consumption comparable with typical US diets (Table 2).

Dietary intervention

Dietitian counselling and provision of study foods. The dietitian met with each study participant for 2 h at the initial visit to administer a diet assessment tool, provide tailored dietary advice, review all diet education materials and provide a 2-week supply of food. Participants attended six 45-min follow-up diet counselling sessions at 2-week intervals throughout the 12-week intervention period. At each follow-up session, participants selected a 2-week supply of prepared and unprepared study foods (sufficient for approximately two-thirds of their energy needs) from a fixed food list (Table 1). Study foods were prepared by the UNC Metabolic Kitchen and packed into 57 litre rolling coolers with ice packs for easy transport.

Self-monitoring. Participants completed a 3 d food record for the second study visit and a daily food checklist for all subsequent visits. The daily food checklist imposed less

participant burden than food records, while still contributing to dietary adherence through daily self-monitoring of consumption of key study foods, such as fish, flaxseed and oils. Each week, the subjects rated their adherence to the diet for the week on a scale of 1 ('excellent') to 5 ('poor'). This subjective score allowed the participant to reflect on and report difficulties in following the diet and helped the dietitian address individual challenges to dietary adherence.

Web-based diet education materials. The following intervention-specific education materials were available to the participants through a password-protected website developed by the Nutrition Applications for Health Communications and Interventions Core of the UNC School of Public Health Nutrition Obesity Research Center: (1) Diet Guidelines; (2) Food Lists; (3) Seven-day Meal Plan; (4) Reading Food Labels Guide; (5) Grocery Shopping Guides; and (6) Dining Out Guide. In addition, more than fifty recipes using study-provided foods and/or meeting study guidelines were included on the intervention website.

The diet guidelines informed participants of specific dietary changes required. The food list complemented the diet guidelines by categorising more than 270 foods into 'allowed', 'limit' and 'avoid' for easy reference. The 7 d meal plans provided practical guidance to achieve the nutrient targets and were assigned to each participant according to their baseline BMI and calculated for weight maintenance (example meal plan in Supplementary Appendix 2, available online).

'Reading food labels' is an activity booklet that illustrated how to identify high-LA foods by reading food ingredients lists. Grocery shopping guides, which list low-LA brand-name foods in all major food categories at ten local grocery stores, assisted participants in obtaining low-LA foods for meals prepared at home. The dining out guide assisted in identification of low-LA foods at local restaurants.

Assessment of diet adherence

Multiple 24 h dietary recall assessment. The UNC Nutrition and Obesity Research Center Diet, Physical Activity and Body Composition in Human Populations Core (NORC) collected participant dietary intake data using six unannounced telephone-administered 24 h recalls per subject: three were administered prior to the intervention (two weekdays, one weekend day) and three were administered in the final 6 weeks of the 12-week intervention (two weekdays, one weekend day). Nutrient values were estimated using the Nutritional Data System for Research (NDSR) software version 2009–2010, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN⁽²³⁾. Because the study-provided foods contained markedly different LA, ALA, AA, EPA and DHA contents than commonly available US foods, the fatty acid contents of study-provided foods were reconciled with the NDSR database in order to improve accuracy of the diet recalls. This required creation of custom user-recipe projects matched to one-tenth of the analysed, study-provided foods. An effort was also made to reconcile the fatty acid content of many of the foods consumed outside the home by calling local eateries. Despite these efforts, the

LA content of some of the low-LA foods recommended for grocery shopping and dining out were not able to be reconciled with the NDSR database.

Erythrocyte fatty acid analysis. Erythrocytes were obtained from the EDTA blood samples after the plasma and buffy coat were removed. Following Bligh/Dyer extraction⁽²³⁾, erythrocyte aliquots were heated at 100°C for 1 h with methanol containing 14% boron trifluoride to generate fatty acid methyl esters. These were then extracted into hexane and analysed with a flame ionisation detector gas chromatograph (Agilent 6890 Plus LAN; Agilent Technologies) equipped with a 30-m DBFFAP (nitroterephthalic acid modified polyethylene glycol) capillary column. Fatty acids were identified through comparison with a standard fatty acid methyl ester mixture (GLC-462). Values are expressed as percentages of total erythrocyte fatty acids.

Data analysis

Because of the small sample size and non-normal distributions of dietary intake variables and erythrocyte fatty acids, non-parametric analyses were performed. Median values and inter-quartile ranges (IQR) for targeted fatty acids were calculated for both groups. Pre-to-intra-intervention and between-group comparisons were made with the Wilcoxon signed-rank test for matched pairs and the Wilcoxon rank-sum test, respectively. *P* values <0.05 were considered significant.

Results

A total of sixty-seven subjects were randomised to either the H3-L6 diet (*n* 33) or the L6 diet (*n* 34) (Table 2). Of these, eleven participants dropped out of the intervention after randomisation (four in the H3-L6 diet group, seven in the L6 diet group) due to trauma unrelated to diet (*n* 1), personal conflicts (*n* 2), disqualification after randomisation (pregnancy or diabetes diagnosis) (*n* 3) or dislike of the assigned diet (*n* 5). One subject who completed all other aspects of the study could not be reached by telephone to complete the intra-intervention 24 h recall assessment. The non-completers (*n* 11) and completers with pre- and intra-intervention diet data (*n* 55) had no significant differences in baseline intakes of any target nutrients (*P*>0.05; Table 3).

Median nutrient intakes and IQR for the fifty-five participants with the pre- and intra-intervention 24-h recall data (*n* 28 for L6 diet, *n* 27 for H3-L6 diet) are presented in Table 3. There were no significant pre-to-post intervention changes in the consumption of total fat, carbohydrate, protein or total energy in either diet group. For the L6 group, dietary LA declined from 7.4 en% (IQR 5.7–9.6) to 2.4 en% (IQR 2.0–2.9). The between-subject variability of dietary LA intake by diet group is illustrated in Fig. 1. Total PUFA declined from 8.1 en% (IQR 6.4–10.6) to 3.5 en% (IQR 3.1–4.3) and SFA increased from 10.5 en% (IQR 9.1–11.9) to 14.0 en% (IQR 12.1–17.2). Intakes of *n*-3 PUFA did not change for the L6 group. For the H3-L6 diet group, LA declined from 6.4 en% (IQR 5.3–7.4) to 2.5 en% (IQR 2.2–3.9). ALA increased from 0.6 en% (IQR 0.5–0.9) to 1.6 en%

Table 3. Diet intervention fatty acid targets and comparison of pre-intra intervention nutrient intakes from 24 h dietary recall* (Medians and 25–75% percentiles)

Variable	Diet targets		Changes pre–post diet intervention											
	L6	H3-L6	L6 (<i>n</i> 28)				H3-L6 (<i>n</i> 27)				Between diets			
			Pre	Median	25–75%	Intra	Pre	Median	25–75%	Intra	Pre	Intra	<i>P</i>	
Total energy (kJ)	N/A	N/A	8355	7778	59.4, 9201	0.52	7142	5761, 7866	6678	5410, 8196	0.89	0.03	0.09	
Total fat (en%)	32	32	33.6	30.4	26.8, 34.3	0.05	33.4	29.1, 36.4	30.7	27.3, 34.0	0.08	0.38	0.84	
Total protein (en%)	18	18	15.7	15.2	13.7, 17.0	0.13	16.1	13.5, 19.6	17.2	15.1, 20.0	0.25	0.62	0.01	
Total SFA (en%)	13	13	10.5	14.0	9.1, 11.9	<0.001	10.5	9.8, 11.7	12.9	9.9, 14.5	0.16	0.99	0.06	
Total <i>trans</i> (en%)	<0.5	<0.5	0.9	0.6	0.7, 1.2	0.005	1.1	0.9, 1.4	0.5	0.3, 0.7	<0.001	0.07	0.06	
Total MUFA (en%)	16	14	11.8	10.4	10.1, 13.2	0.008	12.1	11.0, 13.5	9.3	8.4, 11.4	<0.001	0.82	0.51	
Total PUFA (en%)	2.5	4.5	8.1	3.5	3.1, 4.3	<0.001	7.4	5.9, 8.5	6.0	5.1, 7.1	0.1	0.05	<0.001	
LA 18:2 (en%)	≤2.5	≤2.5	7.4	2.4	5.7, 9.6	<0.001	6.4	5.3, 7.4	2.5	2.2, 3.9	<0.001	0.03	0.15	
ALA 18:3 (en%)	0.6	>1.5	0.7	0.7	0.6, 0.9	0.96	0.6	0.5, 0.9	1.6	1.3, 2.0	<0.001	0.32	<0.001	
AA 20:4 (mg)	60	150	106	48†	57, 159	<0.001	110	66, 176	114†	69, 195	0.75	0.64	<0.001	
EPA + DHA (mg)	125	>1000	43	76†	25, 73	0.32	47	20, 71	1482†	374, 2558	<0.001	0.96	<0.001	

L6, average *n*-3, low-*n*-6 diet; H3-L6, high-*n*-3, low-*n*-6 diet; N/A, not applicable; en%, percentage of food energy; LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid.

* Between-subject and between-diet comparisons were calculated with the Wilcoxon signed-rank test and Mann–Whitney *U* test, respectively.

† Indicates that intra-intervention 24 h recall database contained fewer missing values for *n*-6 AA, EPA and DHA due to chemical analysis of relevant study foods.

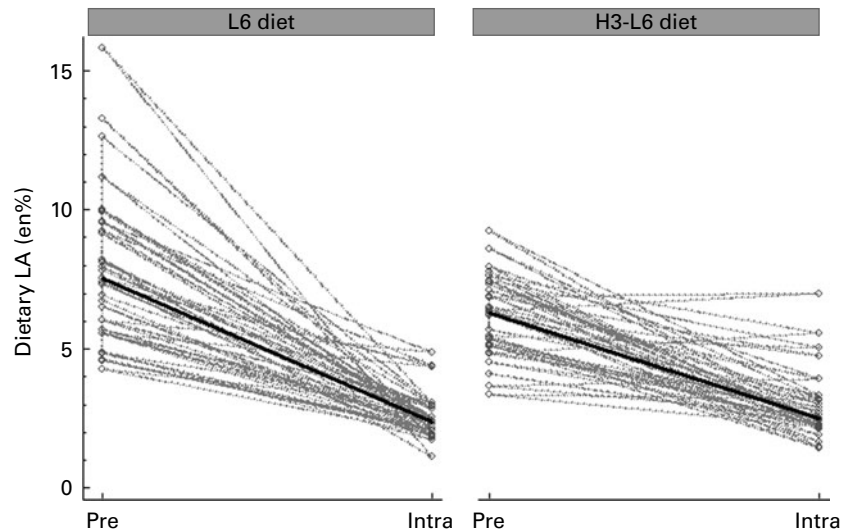


Fig. 1. Dietary linoleic acid (LA) in the pre-intervention (Pre) and intra-intervention (Intra) periods. L6, low-*n*-6 diet; H3-L6, high-*n*-3 low-*n*-6 diet; en%, percentage of food energy. —, Median dietary LA intake.

(IQR 1.3–2.0). Daily EPA + DHA consumption increased from 47 mg (IQR 20–71) to 1482 mg (IQR 374–2558).

Median erythrocyte fatty acids and IQR for the fifty-two participants with pre- and post-intervention data (n 27 for L6 diet, n 25 for H3-L6 diet) are presented in Table 4. For the L6 group, erythrocyte LA declined (-13.6% , $P < 0.01$), and EPA ($+51.3\%$, $P < 0.01$), DHA ($+18.8\%$, $P < 0.01$) and total SFA ($+3.0\%$, $P = 0.04$) increased. For the H3-L6 diet group, LA (-12.6% , $P < 0.01$) and AA (-14.1% , $P < 0.01$) declined, and EPA ($+27.4\%$, $P < 0.01$) and DHA ($+80\%$, $P < 0.01$) increased. Substantial within-group variability in EPA and DHA intakes were noted (Fig. 2).

Discussion

Here, we showed that dietary LA can be markedly reduced, with or without a concurrent increase in *n*-3 PUFA, in a free-living adult population using an integrated diet method. To our knowledge, this is the first demonstration that dietary *n*-6 LA can be lowered to amounts consistent with historical US⁽²⁾ or evolutionary diets⁽³⁾ in an outpatient setting.

Dietary *n*-6 PUFA lowering for 12-weeks reduced the LA content and increased the EPA and DHA content of the erythrocytes, without significantly altering the proportions of *n*-6 AA. Although it is often speculated that high *n*-6 LA intakes induce inflammation by increasing the synthesis and subsequent metabolism of AA into inflammatory derivatives^(9,24), there are currently no human data supporting the proposition that dietary LA substantially alters the abundance or metabolism of AA in human subjects⁽²⁵⁾. The present finding that concurrent dietary lowering of both *n*-6 LA and AA did not significantly reduce the AA content of erythrocytes is consistent with the idea that dietary LA has little or no effect on tissue AA content. Importantly, however, the 12-week duration of dietary LA-lowering employed here may not have been sufficient to achieve the steady-state concentrations of erythrocyte AA due to the high LA content of the adipose

tissue; LA presently accounts for about 15% of total fatty acids in the adipose tissue of the US population⁽²⁶⁾, compared with only 6% in 1961^(27,28). Because the adipose tissue PUFA have a slow rate of turnover^(29,30) and mobilisation of LA from adipose tissue may attenuate changes in circulating PUFA, dietary LA lowering may need to be maintained for relatively long periods of time to elicit maximal changes in the AA content of the erythrocytes and other circulating lipid pools.

Dietary *n*-6 PUFA lowering for 12 weeks did produce significant increases in the EPA ($+51\%$) and DHA ($+19\%$) content of the erythrocytes, suggesting that high-LA diets may interfere with the synthesis and/or accumulation of EPA and DHA in human tissues. Dietary LA (18:2*n*-6) may reduce EPA and DHA in human tissues by: (1) impairing enzymatic conversion of *n*-3 ALA (18:3*n*-3) to 18:4*n*-3 (the precursor to EPA)⁽³¹⁾; (2) impairing conversion of 24:5*n*-3 to 24:6*n*-3⁽³²⁾ (the precursor to DHA)⁽³³⁾; and (3) competing with *n*-3 EPA and DHA (and *n*-6 AA) for esterification into the sn-2 position of phospholipids⁽³⁴⁾. It is generally believed that the synthesis of DHA from dietary ALA is not sufficient to maintain adequate DHA in human subjects⁽³⁵⁾. The present finding of increased EPA and DHA accumulation with dietary *n*-6 lowering indicates that the DHA adequacy of human tissues may depend on background *n*-6 PUFA consumption. Importantly, however, erythrocyte fatty acid data cannot inform whether observed increases in EPA and DHA are due to increased elongation/desaturation of *n*-3 ALA, reduced competition with *n*-6 LA (and *n*-6 AA) for esterification or a combination of both. Tracer studies comparing the kinetics of *n*-3 ALA elongation/desaturation in high- and low-LA diets are warranted.

The H3-L6 diet produced marked increases in erythrocyte *n*-3 EPA and DHA and a reduction in *n*-6 AA, compared with the L6 diet; these changes were probably attributable to consumption of preformed EPA and DHA from the seafood. The observed reduction in erythrocyte AA may be due to

Table 4. Pre- and intra-intervention erythrocyte fatty acids* (Medians and 25–75% percentiles)

Variable (% by weight)	Changes pre–post diet intervention														
	Low- <i>n</i> -6 (<i>n</i> 27)						High- <i>n</i> -3, low- <i>n</i> -6 (<i>n</i> 25)								
	Pre			Post			Pre			Post					
	Median	25–75%	Median	25–75%	Median	25–75%	Median	25–75%	Median	25–75%	Median	25–75%			
LA	12.16	10.99, 12.99	10.51	9.50, 11.08	11.64	10.27, 12.56	10.17	8.76, 10.77	<0.001	–13.6	<0.001	–12.6	<0.001	0.126	0.151
AA	14.15	12.12, 15.03	13.07	12.67, 14.64	14.31	12.73, 15.22	12.29	11.60, 13.03	0.53	–7.6	0.001	–14.1	0.001	0.447	0.013
ALA	0.21	0.19, 0.26	0.25	0.18, 0.31	0.20	0.17, 0.23	0.32	0.23, 0.38	0.35	+19.1	0.001	+60.0	<0.001	0.341	0.028
EPA	0.39	0.29, 0.50	0.59	0.40, 0.79	0.38	0.32, 0.54	1.42	1.00, 2.02	<0.001	+51.3	<0.001	+273.7	<0.001	0.721	<0.001
DHA	3.30	2.66, 3.98	3.92	3.04, 4.97	3.78	3.21, 4.26	6.79	5.72, 8.05	0.001	+18.8	0.001	+79.6	<0.001	0.080	<0.001
Total MUFA	19.11	18.15, 20.18	19.52	18.87, 20.36	18.73	18.25, 19.84	18.54	18.26, 19.87	0.16	+2.2	0.60	–1.0	0.60	0.492	0.074
Total SFA	42.95	41.74, 44.92	44.24	42.24, 45.17	42.54	41.42, 44.92	42.71	41.67, 44.78	0.04	+3.0	0.76	+0.4	0.76	0.905	0.167

LA, linoleic acid; AA, arachidonic acid; ALA, α -linolenic acid.

*Between-subject and between-diet comparisons were calculated with the Wilcoxon signed-rank test and Mann–Whitney *U* test, respectively.

increased competition with *n*-3 EPA and DHA for esterification into the phospholipid sn-2 position, reduced elongation/desaturation of *n*-6 LA or a combination of both.

Although the H3-L6 group achieved the median EPA + DHA intake target of ≥ 1000 mg/d (Fig. 1), there was considerable within-group variability that was probably attributable to aversion to fish in a subset of the participants. H3-L6 dieters who were unwilling to consume at least one serving of seafood per d were encouraged to consume high-EPA + DHA fish ‘as often as possible’ and to continue all other aspects of the intervention. The use of only seafood, rather than *n*-3 supplements, to deliver EPA + DHA is a unique aspect of the present study. Some of the EPA and DHA present in seafood are incorporated in multiple phospholipid classes (e.g. phosphatidylethanolamine, phosphatidylcholine and plasmalogens), which may have different absorption properties and biological activities than the TAG and ethyl ester forms present in refined fish oils and medications^(36,37). Seafood also contains vitamins, minerals and other nutrients that affect the bioactivities of accompanying fatty acids⁽³⁸⁾. However, future feeding trials may benefit by providing *n*-3 supplements to participants unwilling or unable to eat seafood on a daily basis in order to provide more uniform amounts of EPA + DHA.

Limitations

Limitations of 24 h recalls for assessing nutrient intakes include the dependence on self-reported data and under-reporting^(39,40). However, multiple 24 h recalls have been validated with doubly labelled water and are more highly correlated with present energy intake than FFQ and diet history questionnaires^(2,41–46). The 24 h diet recall method is considered a valid tool for estimating dietary intake and is frequently used to validate new methods of diet assessment^(47,48). In addition, this method allowed us to update the nutrient database for the specific study foods and recipes for a more accurate reflection of study food intake during the diet intervention. The NDSR contains detailed fatty acid data, and our efforts to reconcile the fatty acid contents of study-provided foods with the NDSR database probably improved the accuracy of the diet recalls.

Some of the low-LA foods recommended for purchase were not able to be reconciled with the NDSR database and, therefore, our 24 h recall data may overestimate LA intakes during the intervention in both diet groups. Because fatty acid values from direct laboratory testing were reconciled with the NDSR database for all study-provided animal foods, and these study foods had fewer zero values for AA, EPA and DHA than corresponding foods in the NDSR database used in the pre-intervention phase, apparent increases in AA, EPA and DHA may have been overestimated. The observed reduction in erythrocyte LA in both groups and marked increase in erythrocyte EPA and DHA in the H3-L6 group provide further evidence of adherence to the study interventions, but cannot quantify whether the nutrient intake targets were achieved. Finally, because this population with chronic headaches may have been more adherent to the study diets due to the potential for pain relief, the magnitudes of the observed

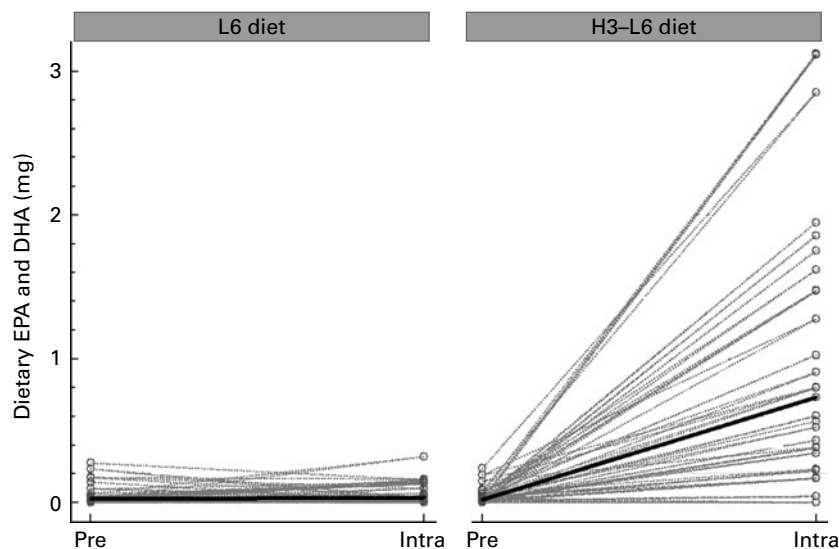


Fig. 2. Dietary EPA+DHA in the pre-intervention (Pre) and intra-intervention (Intra) periods. L6, low-*n*-6 diet; H3-L6, high-*n*-3 low-*n*-6 diet. —, Median dietary EPA+DHA intake.

dietary changes are not necessarily generalisable to non-pain populations.

Conclusion

Dietary LA can be lowered to ≤ 2.5 en%, with or without concurrent increases in dietary *n*-3 PUFA, in an outpatient clinical trial setting using this integrated diet method. These diet methods are intended to serve as a template for future human trials in other clinical populations to evaluate the effects of lowering dietary LA.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114512005181>

Acknowledgements

The authors declare that they have no competing interests. The authors would like to thank the following individuals for their research assistance: Marjorie Busby for expertise with study design; Gus Swenson for study food production management; Dietetic Technicians Clarence Mayo, Jim Howerton and Derrick Williams for preparation of study foods; Olafur Palsson, Beth Fowler, Carol Carr, Regina McCoy and Tim McCaskill (programmer) for design and functionality of the study website; Jim Loewke for erythrocyte and nutrient composition analysis and Duk Hyun for nutrient composition analyses; Chanee Lynch for study coordination; and Rebecca Coble, Amit Ringel, Sharon Majchrzak-Hong and Joseph Graciosa for general research assistance. B. A. M. was the lead dietitian, developed the dietary intervention methods, provided nutrition counselling to research subjects and contributed to the initial draft and revisions of the manuscript. C. E. R. was the co-principal investigator and contributed to the

hypotheses, grant application, study design and implementation and interpretation of the initial draft and revisions of the manuscript. K. R. F. contributed to the overall trial development and conduct, performed the statistical analysis and revised the manuscript. D. Z. assisted with manuscript preparation and statistical analysis. M. M. led the NORC research team in creating custom user recipes, collecting the 24 h dietary recalls and revised the manuscript. J. R. H. contributed to the study design and interpretation and revised the manuscript. J. D. M. was the co-principal investigator and the primary study clinician and contributed to all aspects of the project. All authors contributed to the intellectual content of the manuscript. The authors gratefully acknowledge funding support for the present trial from the Mayday Fund (primary source); the North Carolina Clinical and Translational Sciences Institute (grant UL1RR025747, NCRR, NIH); the UNC Nutrition Obesity Research Center, CHAI Core and Nutrition Epidemiology Core (grant DK056350, NIDDK, NIH); the UNC Research Fellowship in Complementary and Alternative Medicine (grant T32-AT003378, NCCAM, NIH); and the Intramural Research Program of the National Institute on Alcohol Abuse and Alcoholism, NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Mayday Fund or the National Institutes of Health.

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