Supplementation with N-3 Long-Chain Polyunsaturated Fatty Acids or Olive Oil in Men and Women with Renal Disease Induces Differential Changes in the DNA Methylation of FADS2 and ELOVL5 in Peripheral Blood Mononuclear Cells



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Abstract

Background: Studies in animal models and in cultured cells have shown that fatty acids can induce alterations in the DNA methylation of specific genes. There have been no studies of the effects of fatty acid supplementation on the epigenetic regulation of genes in adult humans.

Methods and Results: We investigated the effect of supplementing renal patients with 4 g daily of either n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) or olive oil (OO) for 8 weeks on the methylation status of individual CpG loci in the 5' regulatory region of genes involved in PUFA biosynthesis in peripheral blood mononuclear cells from men and women (aged 53 to 63 years). OO and n-3 LCPUFA each altered (>10% difference in methylation) 2/22 fatty acid desaturase (FADS)-2 CpGs, while n-3 LCPUFA, but not OO, altered (>10%) 1/12 ELOVL5 CpGs in men. OO altered (>6%) 8/22 FADS2 CpGs and (>3%) 3/12 elongase (ELOVL)-5 CpGs, while n-3 LCPUFA altered (>5%) 3/22 FADS2 CpGs and 2/12 (>3%) ELOVL5 CpGs in women. FADS1 or ELOVL2 methylation was unchanged. The n-3 PUFA supplementation findings were replicated in blood DNA from healthy adults (aged 23 to 30 years). The methylation status of the altered CpGs in FADS2 and ELOVL5 was associated negatively with the level of their transcripts.

Conclusions: These findings show that modest fatty acid supplementation can induce altered methylation of specific CpG loci in adult humans, contingent on the nature of the supplement and on sex. This has implications for understanding the effect of fatty acids on PUFA metabolism and cell function.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

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Introduction

Epigenetics refers to a group of heterogeneous, but interrelated processes that regulate transcription without changing the DNA nucleotide sequence. Specifically, epigenetics involves methylation at the 5' position of cytosine bases in CpG dinucleotide pairs, covalent modifications of histones, or the activities of non-coding RNA species [1]. Although the DNA methylation of some gene promoters, for example those involved in cell differentiation or imprinting, is induced in early life and persists throughout the life course, other DNA methylation marks appear to be more plastic [2], particularly during periods of rapid growth [3]. Altered epigenetic regulation by DNA methylation of specific genes has been implicated as a causal factor in a number of noncommunicable diseases [4,5]. Genes that retain epigenetic plasticity may respond to environmental inputs, including nutrition, and so may alter gene and cell function. Thus

	Male		Female	
	Study 1 cohort			
Treatment	n-3 LCPUFA	00	n-3 LCPUFA	00
n	8	8	6	7
Age (years)	55±4	61±3	53±6	55±4
BMI (kg/m²)	25.7±1.0	27.2±1.4	28.7±2.6	29.5±2.8
eGRF (ml/min/1.73 m ²⁾	37.6±4.0	34.9±2.4	36.5±4.0	32.7±3.9
Cholesterol (mmol/l)	5.1±0.2	4.6±0.2	4.8±0.4	4.7±0.2
Triglyceride (mmol/l)	2.4±0.3	1.7±0.2	1.1±0.1	1.8±0.5
Creatinine (µmol/l)	209±22	192±16	151±15	163±17
Glucose (mmol/l)	5.0±0.2	5.0±0.2	4.5±0.3	5.1±0.3
HOMA-IR	3.0±0.4	2.4±0.3	2.3±0.8	3.2±1.0
	Study 2 cohort			
n	8		12	
Age (years)	23±1		30±4	
BMI (kg/m²)	22.6±0.5		21.5±0.5	

 Table 1. Subject characteristics at baseline.

Values are mean \pm SEM. n-3 LCPUFA, fish oil; OO, olive oil; BMI, body mass index; eGRF, estimated glomerular filtration rate; HOMA-IR, homeostatic model assessment of insulin resistance. Comparisons within male and female subjects by Student's unpaired t test showed no significant differences (P>0.05) between treatment groups. doi:10.1371/journal.pone.0109896.t001

understanding the impact of nutrient intakes on the epigenome has important implications for dietary choices in relation to health.

Dietary fat intake can modify capacity for polyunsaturated fatty acid (PUFA) by changing the mRNA expression of $\Delta 6$ (D6d) and $\Delta 5$ (D5d) desaturases [6,7]. Although product inhibition is likely to be involved, the underlying mechanism has not been welldescribed. Changes in dietary fatty acid intake can alter the activity of the PUFA biosynthesis pathway in rodent models via changes in the epigenetic regulation of key genes. Increasing dietary α -linolenic acid content during pregnancy and lactation in mice increased the average methylation of the fatty acid desaturase (Fads)-2, which encodes D6d, promoter and of intron 1 by up to 2% in the liver of dams [8,9]. Feeding pregnant rats diets containing different amounts of saturated or n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) induced hypermethylation of specific CpG loci in the Fads2 promoter and decreased mRNA expression in the liver of the adult offspring. This was accompanied by decreased proportions of docosahexaenoic acid (DHA) and arachidonic acid in membrane and plasma phospholipids [10]. Feeding adult female rats a fish oil-enriched diet for 9 weeks also induced lower Fads2 mRNA expression and increased methylation of specific CpG loci in the Fads2 promoter [10]. These changes were reversed when the animals were switched to a soybean oil-base diet [10]. Feeding dams either 7% and 21% (w/ w) safflower oil, hydrogenated soybean oil, butter or fish oil induced increased methylation of specific CpG loci in the Fads2 promoter and decreased its expression in aortae in the adult offspring [11]. Mutation of one CpG locus that was hypermethylated in both the liver and aortae of these offspring, which is located within an estrogen receptor response element, decreased the activity of the Fads2 promoter [11]. This indicates that at least some of the hypermethylated loci were involved directly in the regulation of Fads2 transcription. Together, these findings support the suggestion that variation in the fatty acid supply during development can induce persistent changes in the epigenetic regulation of LCPUFA biosynthesis.

To our knowledge, no study has examined the effects of dietary fatty acid intake on the epigenetic regulation of genes involved in PUFA metabolism in adult humans. In this study, we tested the hypothesis that dietary supplementation with preparations containing either predominately n-9 monounsaturated fatty acids (olive oil; OO) or n-3 LCPUFA induces differential changes in the DNA methylation of genes involved in LCPUFA biosynthesis. We measured methylation status of individual CpG loci in the 5' regulatory region of FADS2, FADS1 (which encodes D5d) and elongase (ELOVL)-5 and ELOVL2 (which encode elongase 5 and elongase 2, respectively) in DNA extracted from peripheral blood mononuclear cells (PBMCs) isolated from patients with chronic renal disease. We also tested whether any induced changes in DNA methylation were related to the level of mRNA and whether any effects of n-3 LCPUFA on the DNA methylation of genes involved in LCPUFA biosynthesis could be replicated in blood from healthy adults.

Subjects and Methods

Ethical statement

The studies were conducted according to the principles expressed in the Declaration of Helsinki. Study 1 was approved by the Royal Perth Hospital Ethics Committee (EC2004/045) and is registered on the Australian Clinical Trials Register (ACTRN012605555588640). Subjects gave informed, written consent. Study 2 received ethical approval from the National Research Ethics Service Committee, South Central – Berkshire (REC reference number 11/SC/0384). Participants provided informed, written consent.

Dietary intervention trials and sample collection

The samples used in this study were derived from specimens collected in two dietary intervention studies, one in patients with chronic kidney disease at the University of Western Australia

		Α	В	
			_	
5' -	-1340	-1337 GCC <u>CG</u> TTTGACCATCTCTCCAATCTCAGGCTCTCCATTTTCAAGTGAGATGTAATAATA	5' -1037 -1040 AAA <u>CC</u> CTAAGAACAGTGCCTGGCACACAGTAAGTGCTTTATAAAGTGTTTGTT	AA
		-1278	-980 TAAAATTTTGGACCTAAACTCTGGGTCTCTTCAGGACTGCAACAGCTTTGTAACTGGC	AA
	-1280	TG <u>CG</u> TCCTGTTTACCTCTCAGCCTGTGATGAGAATCTAATGATTAGGGTGTGCTAGCACA	-905 -920 CCCCACTTTTAGGTGCGTTCCCACTCCTCTAAAACCCAGAGATCTAAATGCCAAATCT	ст
-	-1220	CAGGCACCTGTAAATCCCATTGAAATCTGAGGGGCCCTATAACTCCTCTAGTGATTCCCAA	-830 -860 CTGCTTAAAAAGTCTCCCAGGGCTCCTAGG <u>CG</u> CCTCCAGGCTAGAACAGAAATGCCTC	AG
	-1160	-1156 -1119 -1112 -1101 GCCTCGTGCACCCCACCCTCTCTGTTCATCCTTACTTCCCACGTGTCCCGTTAGCCCTCC	-800 CTTGAAGACCCAGGCTTTTCAGGTGAAACACCTAAGGGTCAGGAGAC <u>G</u> CTAGGATCAT	CA
		-1071 -1067 -1056	-740 CTCAAGGATCCCAGTGAATTTTTCCAAAATACAATAAAAATAAAAAACAAAAAGAGGCA	AA
-	-1100	<u>G</u> GATGCAGTCAGGCCCATTTCCCCCAGGA <u>CG</u> CC <u>CG</u> GCACTAAGC <u>CG</u> CCCCATCCAGCTGG	-680 CAGGGTTATAAAAATTGTGGGGCATTTTAAATGTTTCATTGAACAAATTAAAGCATTA -586	AC
	-1040	-1013 GGTCTGAGGGGCCTGTCTCTTGCCCCACGCCTAAAAGACCTAACCCTCCTCCTATCCCTT	-620 AGCCCTCCCCCAACCACCAAGCCCAAGAGACCCGTAAATATGCTGTTCACAAGATA	AC
		-980 -975	-560 TGCAACTTTCAAGGGCTCTCAGGCTGCTACTTCGGGCAGCACAATTGGCGGCACGACG -464 -451	ŦG
-	-980	<u>CGATACCGCAGTCTTTATTTGCTGGAGTCTGCACAACATCACTGCCCAATGATGTGTCTG</u>	-500 GCAAGCAGGCAGTAGTTTCCAACCCTGGAGGGTCAGCGTCTGGAGACCCCCGGCCAAGG -418-416-412-408 -398 -392	CA
	-920	-914 CTTCCA <u>CG</u> ATTCCCAAAGAGACTGAGCTTACTGAGACCAGGGCAAGGAC <u>CGCG</u> CCAGTTC	-440 TCCACAGCCTAAAGATGATGTCCCGCGACCGCCCGGGCAGCCTCGTGCACGGAAAAAACC -375 -369 -354 -345	ГC
		-855 -817 E2F -806	-380 AACCCCGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	TT 63
	-860	CTCATCGCCCCCTTCCTCTGCCTTCCCCTCCCCTCACCGCAGCCATGGCGCCCA	-320 CACTCCTGCAGCGCGCCCCGCACCCAGGGCCTGCACTAGAACCGCTGTTCCTACCGC -260 -243 -240 -238 -231 -224 -213 -207 -20	<u>G</u> G 4
-	-800	EGR1 -775 E2F3 GACCAAGAAAGCAGAGCAGAGGGTTCCGCAATTCTTTTCTAAGATTGTCTGACTAGAGGGT	-260 CCCCCTGGGAGCCAACGCCGCGATGCCCGCCTGACGTCAGGAAGTCGAATCCGGCG	GC
	-740	$\begin{array}{c} -718^{\text{C}/\text{EBP}} \beta & -686 \\ \text{TCAAAGCCCTCTAATCCAAGGCCGCCTGTGTGTCTCTAGGGAGGTTGCAGAAAGGCGCCAG} \end{array}$		
	c 00	-669 -667		
	-680	AATGTGGATGG <u>CGCG</u> GACAATGTGGGATAC		
	-680	ANTGTGGATGG <u>CGCG</u> GACAATGTGGGATAC 3'		
	-680	antgtggatgg <u>cggg</u> gacaatgtggggatac 3' C	D	
E'	-680	aatgtggatgg <u>cgg</u> gacaatgtgggatac 3'	D	
5'	-740	алтетедатес <u>оссс</u> дасалтетедедатас 3' Сстсалаладдалалаладалалаладалталатттесстасатталатасс <u>сс</u> асте	5 -539 -533 -501 -491 -500 -501 -491 -500 -501 -491 -500 -500 -500 -500 -500 -500 -500 -50	481 C
5'	-740	алтотодатод <u>о со со</u>	D -539 -533 -508 -501 -491 - -540 ccgcgaacgegaacagagactcttgcccaggccgttatcccaacctatccgecttccca -480 gcacccccagagagaacccaacccaaccccctaaacctaagaaacccagactgeaacc -376	481 C T
5′	-740	алтетедатес <u>ассесе</u> дасалтетедедатас 3' ССС	5' -539 -533 -501 -491 - -540 ccccccaccacccaccccaccccaccccaccccaccc	481 C T A
5'	-740 -680 -620		D 5' -539 -533 -508 -501 -491 -490 -480 <u>cccecccacacccccacccccacccccacccccacacccccacccc</u>	481 C T A T
5'	-740 -680 -620 -560		D 5' -539 -533 -509 -501 -491 - -540 CCCCGGGAGCCGGGACAGAGACTCTTGCTCAGGGCCGTTATCCGAACCGACTCTCCCCA -480 CCACCCCCAGAGAAACCCAACCCCCTAAACCTAAGAAACCCAGCCTGCCAAACCC -420 CCACCCCCAGAGAAACCCAACCCCCTAATGTGTGCTCTAAACCCAGCCTGCCAAACCC -376 - -420 CCAGGAAACCAACCCCCTAATGTGTGTGTGCTCTAAACCCAGCCGAAACCC -350 -344 -355 -319 -311 -304 -360 GCAAGACCAGCCGTGCCCCGCCCGCACGATACTGCTTCTCCCCCGCAGCAGCGGCTGCCCGAA -292 -270 -264 -256-283-251-249 -300 CTGGGCAGCCGGGTATTCCTGGGGCCCCCGCCGAAACCC	481 C T A T 3'
5′	-740 -680 -620 -560		D 5' -539 -533 -508 -501 -491 - -540 CCGGGAGCGGGACAGAGACTCTTGCTCAGGGCCGTTATCCGAACTGATCOCCTTCCCC -480 GCACCCCCAGAGAAACCCACCCAACCCCTAAACCTAAGAAACCCAGAGCCCAACTGTA -420 -480 GCACCCCCCGAGAGAACCCACCCCAACCCCTAAGAAACCCCAGCGCAAACCC -420 -376 -376 -420 GCAGGAACAGAGCCATTTCCCCCCTAATGTGTGCTCTCAAACCCAGAGCCCAACTGTA -350 -344 -350 -311 -304 -360 GCAAGACCAGCGTGCCCCCCCGCACGATACCTTCCCCCGCCAGAGCCCCCCGCCCG	481 <u>C</u> T A T <u>3</u> '
5′	-740 -680 -620 -560		D 5' -539 -533 -508 -501 -491 -640 -540 CCGGGGAGCCGGGACAGAGACTCTTGCTCAGGGCCGTTATCCGACATGATCCCCCTCTCCCCCCAGAGAACCCAACCTGCCCCAACCCCCAACCCCCAAACCCCAACCCCAACCCCAACCCC	481 C T A T G 3'
5'	-740 -680 -620 -560 -500 -440		D 5' -539 -533 -509 -501 -491 -6491 -540 <u>ccaecacaecaecaecaecaecaecaecaecaecaecaec</u>	481 C T A T 3'
5′	-740 -680 -560 -500 -440 -380		D 5' -539 -533 -508 -501 -491 -540 CCGGGAGCGGGACAGAGACTCTTGCTCAGGGCGTTATCCCAACTGATCCCCTCCCCACGCGCAACC -429 -480 GCACCCCCGAGAGAACCCACCCCAACCCCCTAACCTAAGAACCCAAGGCGCCAACC -376 -326 -420 GCAGGAACGAGGCCATTTCCCCCCAACCCCCTAACCTAA	481 T A T 3'
5′	-740 -680 -620 -560 -380 -320		D 5' -539 -533 -509 -501 -491 -294 -540 CCGGGAGGGGACAGAGACTCTGCTCAGGCGCGTATCCGACACCAGCCTGGCAAACC -480 CCACCCCAGAGAAACCCAACCCCCTAAACCTAAGAAACCCAGCCTGGCAAACC -376 -420 CCAGGAACGAGGCATTCCCCCCTAATGTGTGCTCTCAACCCAGCGAGCG	481 T A 3'
5'	-740 -680 -620 -560 -380 -320 -260	AATGTGGATGG <u>CGCGG</u> GACAATGTGGGGATAC 3' CC CTCAAAAAAGAAAAAAAGAAAAAAAGAAAAAAAGAATAAATTTGCCTACATTAAAAAGCCGACTG TTAGGTGGATGCACATAGAGGAGAAAAAAAAGAAAAAAGGAATAAATTTGCCTACATTAAAAAAGCAAAGGGGATACAAGGGGGCACACGAAGAAAGGGCTA AR TTAGGTGGGATGCACATAGAGGGAGAAAAGGAAAAGGAATACAGGCAAGGGGGCACACGGAAGAAAGGGCTA AR TTAGGTGGGTTGGATGGGGAAACAGTAGGAATACAGGCAAGGGGGCCACACGAAGAAAGGGCTA AR TCCCAAAATATCAGTGGAAAAGGAAACAGTAGGAGTTGTAAGGCCTAATGAAGGCTTTGATT TCCAAAATATCAGTGGAAAAAAAAAA	D 5' <u>-539 -533</u> <u>-509 -501 -491 -294</u> -540 <u>CCGGGGAGGGGGACAGAGACTCTGCTCCGGCGCGTTATCCGACAGCCGCGCGCG</u>	481 C T A T 3'

Figure 1. Sequences of the 5' regions of (A) FADS2, (B) FADS1, (C) ELOVL5 and (D) ELOVL2 that were analysed by pyrosequencing. Individual CpG loci are indicated by their position relative to the transcription start site (bp) and are underlined. Sequences corresponding to CpG islands are indicated grey shading. doi:10.1371/journal.pone.0109896.g001

(Perth, Australia) (Study 1) and the other in healthy adults at the University of Southampton (Southampton, UK) (Study 2).

The design of the dietary intervention trial in Study 1 has been described in detail elsewhere [12]. The samples used in the current study were from a sub-group of 29 men and women for whom paired before and end of intervention samples were available, who were non-smokers and non-diabetic, with moderate to severe chronic renal impairment (estimated glomerular filtration rate between 15 and 60 ml/min/1.73 m² (normal range ≥90 ml/min/1.73 m²) and serum creatinine less than 350 µmol/1 (normal range 53 to 115 µmol/1). The primary exclusion criteria were use of non-steroidal anti-inflammatory or immunosuppressive medication, consumption of fish oil supplements, consumption of more than 4 alcoholic drinks per day. Six out of the total cohort of 35 subjects only had single samples and so were excluded from the present

analysis. The details of the subjects from whom samples were analysed in the current study are summarised in Table 1.

Subjects consumed 4 g n-3 LCPUFA, eicosapenatenoic acid (EPA; 1.8 g), dosocapentaenoic acid (DPA; 0.2 g) and DHA (1.5 g) ethyl esters in four 1 g capsules (Omacor, Solvay Pharmaceuticals, Pymble, NSW, Australia) or 4 g olive oil (OO) in four 1 g capsules (Cardinal Health Australia, Braeside, Victoria, Australia) each day for 8 weeks. The study had a randomised double blind design. Venous blood samples were collected in the fasting state and differential blood counts were determined as described [12]. PBMCs were isolated as described [13], stored at -80° C until analysed.

The subjects for comparator analyses in Study 2 were the health subjects in a reference group in a study that compared the effect of fish oil supplementation in individuals who were of normal BMI or Table 2. PCR primer sequences.

Primer loc (bp relativ	cation ve to TSS)	PCR primer sequences	
Start	End	Forward (5' to 3')	Reverse (3' to 5')
		FADS1	
-309	-309	GGAGGGTTAGAGTTTGGAGATT	СССССАТАААТСТТАААСААСТСАСАА
-392	-375	AGTGAATGGATTGAGGGGTTAG	СССССАССААААСАТССАСААССТАА
-504	-418	AGTGAATGGATTGAGGGGTTAG	ΑCCCCCACCAAAACATCCACA
-586	-528	AGAGGTAAATAGGGTTATAAAAATTGTG	ССТССААААТТАААААСТАСТАСТАСТТА
-754		AAGAGGTAAATAGGGTTATAAAAATTGTG	ССТССААААТТАААААСТАСТАСТАСТ
-830		AGGGTTTTTAGGAGTTTTTAGGTTAGAA	ACAATTTTTATAACCCTATTTACCTCTTT
-905		AAGAATAGTGTTTGGTATATAGTAAGTG	ΑΑCCTCTCCTAACCCTTAAATATTTCACCT
-1037		AAGAATAGTGTTTGGTATATAGTAAGTG	TCTCCTAACCCTTAAATATTTCACCT
		FADS2	
-1661	1655	GTATGGTGGTTTTGAGGATTGTT	ΑΑΑΑΤΑCTCCCTAATTCTACCTTTCAACTA
-1337		TTGTTGTGAAATTTAGATTGGGTAGG	ССТАААААААТАААССТААСТАСАТ
-1278		TTGTTGTGAAATTTAGATTGGGTAGG	ССТАААААААТАААССТААСТАСАТ
-1156		TTGTTGTGAAATTTAGATTGGGTAGG	ССТАААААААТАААССТААСТАСАТ
-1119	-1101	ATTTGAGGGTTTTATAATTTTTTAGTGAT	ΑCCCTAATCTCAATAAACTCAATCT
-1071	-1056	ATTTGAGGGTTTTATAATTTTTTAGTGAT	CCCTAATCTCAATAAACTCAATCTCTT
-1013	-975	ATTTGAGGGTTTTATAATTTTTTAGTGAT	CCTTACCCTAATCTCAATAAACTCAATC
-914	-855	GGTAGTTTTTATTTGTTGGAGTTTGTAT	AAACCTCTACTCTACTTTCTTAATCT
-817	-775	ATTGAGTTTATTGAGATTAGGGTAAGG	ΑCTTTAAACCCTCTAATCAAACAATCTT
-718	-667	ATTGTTTGATTAGAGGGTTTAAAGTT	AAACTCCAATATCCCACATTAT
-258	-244	AAGATTTTTTTGGGTTAATGGT	ATCCCTAACTTCCCAATACC
-133	-84	AAGATTTTTTTGGGTTAATGGT	ΑΑΑΤCCCTAACTTCCCAATAC
-64	-50	GGGGAGTTTTTATTGGAGGTAA	AATCCCTAACTTCCCAATACC
-18		TGGGGGTATTGGGAAGTTAG	CCCTCCCCAACCTTCTC
		ELOVL 2	
-237	-270	TTTGGGTAGAGGGTGGGTATTTT	CCTCTCCCACAAAAACCT
-292	-350	AGATTGAGTAAATTTGTAGGAATAGAGT	AAACCCCAAAAATACCCACC
-376		AGGAGGTTATAGTTTTGTTTATAGTGAAGA	AAACCCCAAAAATACCCACC
-429		AGGAGGTTATAGTTTTGTTTATAGTGAAGA	ТССАССААААССССАААААТАССС
-481	-508	AGGAGGTTATAGTTTTGTTTATAGTGAAGA	CACATTAAAAAAAAAAAAACTCTATTCCTAC
		ELOVL 5	
-6	-14	GGTTTATTAGGAAGAAAGGGGAAAA	ΑΑCCTAAACCCAAATTAACCCC
-59	-100	GGGAGTTATGGTTATAATAGTTTTGAGT	ΑΤΤΤΤΤΤΤΟΟΟΟΤΤΤΟΤΤΟΟΤΑΑΤ
-134	-185	AGGGAGTTATGGTTATAATAGTTTTGAGT	ΑCCCTAAACTCTAATTTTTTACTACCA
-231	-266	TTTTGTAAGGGAGTTATGGTTATAATAGT	ΑCCCTAAACTCTAATTTTTTACTACC
-269	-316	AGAGTAGAAATGGAGTTGATAGTGG	ΑCCCTAAACTCTAATTTTTTACTACCA
-633	-686	GGAGGTTGTAGTGAGTTGAGA	ΑΑCCTTCATTAACTTTATATCCTACTATT

TSS, transcription start site.

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clinically obese. Subjects men and women of BMI between 18.5 and 25 kg/m², with fasting plasma glucose <5 mmol/l, triacylglycerol <1.0 mmol/l and total cholesterol less than 5 mmol/l who did not consume fish oil or other oil supplements, who did not eat more than one oily fish meal per week, did not have diagnosed diabetes or chronic gastrointestinal problems and were not pregnant or planning to become pregnant within the study period. Subjects were excluded if they used prescribed medicine to control inflammation, to control blood lipids or to control blood pressure. The characteristics of the subjects in Study 2 are detailed in Table 1. A fasting venous blood sample was collected at baseline and an aliquot of whole blood was stored at -20° C. Subjects then received EPAX6000 TG (EPAX, Oslo, Norway) which provided 1.1 g EPA, 0.1 g DPA plus 0.8 g DHA in the form of a triacylglycerol per day for 12 weeks. A second fasting blood sample was collected at the end of the study and an aliquot of whole blood stored at -20° C.

Table 3. Pyrosequencing primers.

Nature Server FADS1 -260 AGTITIGGTTTTAGTTATTA -260 AGTITAGGEGETTAGG -309 GATAGGEGETTAGG -309 GATAGGEGTTAGG -504 AGGETTTTAGGTTGT -504 AGGETTTTAGGTTGT -504 AGGETTTTATAGGTTGT -505 ATTTTAATGATATAGGT -506 ATTTTAAGGTAGGAG -607 GGAATTAATGATAAGGT -608 CTAAACATTCATTGTATGTAAGGT -1037 GGAATTAATGATAAGGATAGGAG -1037 GGAATTAATGATAAGGATAGGAGT -1037 GGAATTAATGGATAGGAT -1037 GGATTTGAAGGATTAAGTGAGT -1138 GGGTTTTTAATTGATAGTGATGT -1139 AAATAACCTAACTACCTACTACT -1130 ATTATCAATCCAACTC -1131 ATTATCAATCAACTCCAACTACT -1132 ATTATCAATCAACTACTACTACT -1134 ATTATCAATCAACTCACT -1135 CCTAATCAACAACTCAATTACT -114 ATTATCAATCAACTACTACTACT -115 CCTAATCAACAACTTAATACT -116 CCTAATCAACAACTACTACA	Starting location (bp relative to TSS)	Primer sequence
-860 AGTTTGGGTTTTAGTTATTA -309 GATAGGGGTTAGG -392 GATAGGGGTTAGG -392 GATAGGGGTTAGG -392 GATAGGGGTTAGGT -394 AGGTTTTTAGTTAGGTGT -586 AGTTTTTTTAGTTATAGT -586 AGTTTTTTTAGTTATAGGTAGGAG -580 CTAAACATTCATAGGTAGGAG -830 CTAAACATTCATGTAACCT -905 AGTTTGTATGGAGTTAATGGTAGGAG -1037 GGAGAATTAATGGTAACGT -1038 AGTTTGGCAGGTGTAGG -1139 AGATGGCTAGGGT -1278 GGTTTTTATTTTAGTGTAGT -1119 AAATAACCTAACTCAACATC -1119 AAATAACCTAACTCAACATC -1119 AAATAACCTAACTCAACAAT -1119 AAATAACCTAACTCAACAAT -1119 AATTGTGTTAGGTAT -1119 AATTGTGTTG -1119 AATTGTGTTGAGT -1119 AATTGTGTTAGGGTTG -1113 GGTAGGTGGTAGGGTT -1114 GGTAGGTGGTAGGGTT -1115 GGTAGTTGGGGTT -1127 GGTA		
-240 ANTITUGUITURA 399 GATTGAGGGTTAGG -392 GGTAGGGGTTAGG -392 GGTAGGGGTTAGG -394 AGGTTITAGTGTA -594 AGTTTATAGGGTAGAG -596 AGTTTATAGGGTAGAGA -586 AGTTTTATAGGGTAGAGA -589 CTAAACATTCTATCTAACCT -905 AGTTGAGGAGAGA -1037 GGGAATTAATGGTAATTTA -1037 AGATGGGTAGGGT -1037 CGGAATTAATGGTAATGGAA -1037 AGATTGGGTAGGGT -1037 AGATTGGGTAGGGT -1037 AGATTGGGTAGGGTT -1138 GGTTTTTATTTTTATAGGGTATGAAT -1199 AAATTAACTAACTCAAAT -1119 AATTACAAACTCCAACT -1013 ATTATACAAACTCCAACT -1013 ATTATACAAACTCCAACT -1119 AATTAGTATTATATTTTTAAT -58 GGTAGGGGGT -617 CCTAATCAACCCAAT -133 GGGTAGGGGGT -134 GGGAAGTTAGGGAGT -135 GGGAAGTTAGGGGATT		
399 GATLARGEGGTTAGG 392 GGTAGGGTTAGG 594 AGGTTTTTAGGTTGT 596 AGTTTTTTATATTATAGT -586 ATTTTTAGGTAGGTTAGGGA 754 ATATTTAGGTAGGTAGGAG 800 CTAAAACATTCTATACT -905 AGTTTGTAATGGTAATTGA -1037 GAGAATTAAATGAGTAAGG -1037 GAGAATTAAATGAGTAAGG -1038 AGTTTGTAAGGTAGGAT -1039 AGATTGGTAGGTAGGAT -1030 TGGTTTGAGGATGTAA -1135 GGGTTTTTTTTATATATAGGAT -1156 GGGTTTTATACTACATCC -1071 CCTCAAACCCAACT -1013 ATATAGCAACTCCAACAAT -1014 ATATGTTTAATGTTATAAGGAT -118 GGTAGAGGGTAAGGTT -258 GATTATTGTAAGGAT -118 GGGAAGTTAGGGATT -292 GGAAGTTAATGTAAGAT -293 GGAAGTTAATGTAAGAT -294 ATAACTCATACTACACAAT -18 GGGAAGTTAGGGATT -292 GAAGTTAATGTAAGATT -293 GAAGTTAGTGAACAT	-260	
-392 GGIAGGGGIIGAGGGII -394 GGIAGGGGIITIAGGITGI -396 AGTITTITTAGTIGT -386 AGTITTITTAGTIGT -380 CTAAACATITGIATCIAACCI -395 GGAGAATIAAATGGGTAAGGA -1037 GGAGAATIAAATGGGTAAGGT -1037 GGAGAATIAAATGGGTAAGGT -1037 GGAGGATTAAATGGGTAGGT -1037 GGATTATAATTGGTAGGT -1037 GGATTATAATTGGTAGGT -1156 GGTTTTTAGTTTAA -1157 GGGTTTATAATTTTAAGTGACATG -1158 GGGTTTTAATTGATAGCTAT -1119 AAATAAACCTAACTACATCC -1011 CCTCAAACCCCAAAT -1013 ATTATGTATGATGGTTTG -118 GGGAAGGGTTAAGGGAT -118 GGGAAGTAGGGAT -118 GGGAAGTTAGGGAT -1292 GGGTATTTGGGGTT -292 GGGTATTTGGGGTT -292 GGGTATTTGGGGT -292 GGGTATTTGGGGT -292 GGGTATTTGGGGT -292 GGGTATTTGGGGT -292 GGGAATTTAGTGGG	-309	GATIGAGGGGTAGG
-594 AGGGTTTTTAGGTGTTG -586 AGTTTTTTATTATAAGT -586 AGTTTTTAGGTATTATTAAGT -587 AGTTTGTATTGGGTAGGAG -830 CTAAAACATTTGTATTGGTAAGTAAGG -905 AGTTTGTAATGGTAATGAG -1037 GGGGAATTAAATGAGTTAAGG -1037 AGGTTTGTAGGTAAGG -1037 AGGTTTAGGTATAATGAGTAAGG -1137 AGGTTTTAGGTATGTAA -1337 AGGTTTATATTTAGTGAGATG -1156 GGTTTTATATTTTTTTAGTGAGT -1157 GGGTTTAACTACACCCAACT -1190 AAAAAACCTAACTACCACAAT -1191 CCTCAAACCCCAACT -1013 ATTATCGTTAAGTGTATTTAG -117 CCTCAACCCCAACT -118 AGGGTTAAGGTATAGTGTA -118 AGGGGTTAAGGTATGGTA -133 GGGTAGGGGGT -258 GGTTTAGGGATTGGTA -134 GGGAAGTAGGGAT -252 GGGTTATATGTGAAGTAGTAGGAT -253 GGGTATAATGGGGT -254 GGGTAGGGGTT -252 GGGTAGTAGGGATTAGTGAAGAGT <	-392	GGTAGGGGTTGAGGTTT
-586 AGTITITITIAATTATTAATTATTAATTAATTAATTAATT	-504	AGGGTTTTTAGGTTGT
754 AATATTAAGGGTTAGGAG -830 CTAAAACATTICTATCIAAGCT -830 AGTTTGTAATTGTAATTTA -965 AGTTTGTAATTGTAATTTA -1037 GGAGAATTAAATGAGTTAAGG -1037 GGAGAATTAAATGAGTTAAGG -1037 AGATTGGGTAGTGTAA -1031 AGATTGGGTAGGGTT -1337 AGATTGGGTAGGGT -1278 GGTTTTATAATTTTTTAGTGAGATG -1196 GGTTTTATAATTTTTTTAGTGAGATG -1197 AAATAAACCTAACTACATCA -1013 ATTATACAAACTCCAACAT -1014 ATTATGTTAATGATGTGTTTG -118 CCTCTAATCAAACACTTAATA -119 AATAATGTTAATGATGTGTTTG -114 ATTATGTTAATGAGGTTAAGGTA -115 GGGTAGAGGGTTAATGGTAT -116 GGGTAGGGGTTAATGGTA -117 CCTCAATCACCCCAACT -118 GGGTAGGGGTTAATGGTA -133 GGGTAGGGGTTAATGGTA -133 GGGTAGGGGGTGT -252 GGGTAGGGGGTT -252 GAAGTTTATGTAATGTAGTGTTTA -252 GAAGTTATGTAATGATAGGTT -252 GAAGTTATGTAATGATAGGTT -254 GAAGTTATGTAATGATAGGTT -255 CTAAAACCAATTGCACCCCCC -376 TATTTTTTTTAGGTATAACCCCACT -481 G	-586	AGTTTTTTTAATTATTAAGT
-830CTAAAACATTICTATICTATCTACCT-905AGTTITGTAATIGTAATTGGTAATTTAA-905GGGAAATTAAATGGTTAAGG-1027FADS2-1661TGGTTITGAGGAATIGTAA-1337AGATIGGTAGGGT-1278GGGTTTTATTTTATTGAGGAATG-1156GGGTTTTATTTTTTAGTGAGGAT-1179AAAATAAACCTAACTACATCC-1119AAAATAAACCCAACT-1013ATTATGATTAGTGTTIG-114CTCTAAACCCAACT-115GGGTTTATAATTTTTAGTGATT-113AGAGGGTTAAAATTAAATCCAACTCAACACT-1013ATTATGATGGTTIG-114CCTCAAACCCAACT-115GGGTAGAGGGGTGT-117GGGTAGGTTAGGATAGGAT-118GGGTAGGTTAGGGAT-220GGGTATTTGGGTT-237GGGTATTTGGGGTT-249AATAACTCTATCTACAACAATT-413GGGAAGTTAGGGTT-292GAGAGTTAGGGTT-292GGGTATTTGGGGTT-292GGGTATTTGGGGTT-292GGGAAGTTAGGGATT-292GGGTATTTGGGGTT-292GGGTATTTGGGGTT-292GGGTATTTGGGGTT-293GAGGTTAGTGGGATTAGGGGT-294ATAACTCTATTCTACAAATT-295GCTAATACCCTAATTAGTGGTTTTA-292GGTATTTGGGG-293GAGAGTTAGGGATTAGGG-294ATAACTCTATTCTACAAATT-295GCTAAAAAATAGGGTTAGGG-296ATAACTCTATTACCCCTC-313GTAAAAAATTAGGGTTAGGG-292GGGTATTAGTGGATATAGCCCTC-293GAGGTTATGATTATTCTACGGG-294ATAAC	-754	AATATTTAAGGGTTAGGAG
-905 AGTITIGTAATTGGAATTAATGAG -1037 GGAGAATTAAATGAGTTAAGG -1037 GGAGAATTAAATGAGTTAAGG -1661 TGGTTTGAGGATGTAA -1337 AGATTGGGTAGGGTT -1278 GGTTTTTAATGTAGGATG -1190 GAAATTAATTTTTTAAGTGAGATG -1191 AAAATAACTAACTACATCC -1071 CCTCAAACCCCAACT -1013 ATTATCAAACTCCAACATA -914 ATTATGTTAATGATGTGTTG -917 CCTCAATCAACAACTCTAAAAT -918 AGAGGGTTAAAGTTTTAATGATGGTA -718 AGAGGGGTGT -64 CCCCAATACCCCCAAA -133 GGGTAAGGAGGTGT -237 GGGTAATTGGGGATT -292 GAAGTTATGTAGTAATGAGAAT -429 AATAACTCTAATTGAAGTAGAAT -429 AATAACTCTAATCCACCCCCA -441 AGAGGATTATGGGG -422 GAAGTTTATTGAGGGAGGGG -538 GGTAGTTAGGGATT -133 GGGTAGGGGATT -134 GGGTAGTGGTG -143 GGGTAGGGGTG -244 GGTAGTAGTAGGGATT -257 GGGTAGTTATGGGGT -260 GAAGTTAGTAATTGTAAGTAGATT -481 AGAGAGTTAGGGG -59 CCTAAACCCAAATTACCCCCC -59	-830	СТААААСАТТТСТАТТСТААССТ
-1037 GGAGAATAAAGGATTAAGG FADS2 FADS2 -1661 TGGTTTTGAGGATGTTAA -1337 AGATTGGGTAGGGT -1278 GGTTTTTATTTTTAAGTGAGATG -119 AAAATAGACTACACTCC -1071 CCTCAAACCCCAACT -1013 ATTATCAAACATCCAACAAAT -914 ATTATCAAACATCCAACAAAT -917 CCTCAAACCCCAACT -118 GGGTGAGGAGGTT -258 GGTTATGGGTAAGGGTT -133 GGGGTAAGGGAGGTGT -148 GGGTAAGGGGGT -237 GGGTAATGGGGTT -242 GGGTATTTGGGGTTAGGGTT -292 GGAAGTTAGGGGTT -292 GGGTATTTGGGGTT -292 GGGTATTTGAGGGAGT -292 GGTATTTGAGGGTT -292 GGTATTTGAGGGTT -292 GGTATTTTGTAGTTATGTAAGT -292 GGTATTTGTAGGGTT -292 GGTATTTGTAGTTTTGTAGGGTT -292 GGTATTTGTAGTTTTGTAGTTTAGT -292 GGTATTTGTAGTTTTTTTTA -292 GTAAAAATTGAGTTTAGTAGGTTA	-905	AGTTTTGTAATTGGTAATTTTA
FADS2 -1661 TGGTTTTGAGGATIGTTAA -1337 AGATTGGGTAGGGTT -1337 AGATTGGGTAGGGTT -1228 GGTTTTTATAGTGAGATG -1156 GGGTTTTATATTTTTTAGTGAT -1119 AAAATAAACCTAACTACATCC -1071 CCTCAAACCCCAACT -1013 ATTATACAAACTCCAACTAAT -914 ATTATGTTAATGATGTTTTG -117 CCTCTAATCAAACAAT -918 AGAGGGTTAAAGTTTTAAT -258 GATTTTTTGGGTTAATGGTA -133 GGGTAGAGGAGTGT -258 GGTATAGGGATT -259 GGTATAGGGATT -251 GGGTAGAGGAGTGT -252 GGAAGTTAGGGATT -253 GGGTATAGGGATT -254 GGGTATGGGGATT -252 GAAGTTTAATTGTAAGTAAGAT -253 GGGTATAGGGATT -254 GGTAATGGGATT -252 GAAGTTTAATTGTAAGTATAGGGT -253 GGTATATGTGTAATTGTAGGAT -254 GAAGTTTAATTGTAGGGTT -255 GCTAAACCCAAATTACCCCAAATT	-1037	GGAGAATTAAATGAGTTAAGG
-1661 TGGTTTGAGGATTGTTAA -1337 AGATTGGTAGGGT -1278 GGTTTTTATTTTAAGTGAGATG -1156 GGGTTTATAATTTTTTAAGTGAGATG -1157 AAAATAAACCTAACTACATCC -1071 CCTCAAACCCCAACT -1013 ATTATACAAATGTTTAATGATGTGTTG -1014 ATTATTACAAACTTAAAAATTAACATACTACAACATC -115 CCTCAAACCCCAACT -1013 ATTATGATAATGTTGTTG -114 ATTATGAACATCTAAACATCTAAAAT -115 CCTCAATCAACACTCAAAAT -116 CCCTAATCAACATCTTAAAA -117 CCTCAATCACCCAAAT -258 GATTTTTTGGGTTAATGGTA -258 GGGATAGGGAGGGTGT -133 GGGATAGGGAGTGGGAT -140 CCCAATCCCCCAAA -18 GGGAAGTTGGGATT -292 GAAGTTTATGTAGTAGGAT -292 GAAGTTTATGTAGGGATT -292 GAAGTTTATTGTAGGGGTTA -292 GAAGTTTATTGTAGGGGTTTA -419 AGAAGTTTTGTTAGGGGTTTA -429 AATAACCTATTCCTACCAAATT -441 GTAAAAAATTGGGGGTTAGGGG		FADS2
-1337 AGATTGGGTAGGGTT -1278 GGTTTTTTAATTGTTTAAGTGATG -1156 GGTTTTATAATTTTTTAAGTGAT -1119 AAAATAAACCTAACTACATCC -1071 CCTCAAACCCCAACT -1013 ATTATACAAACTCCAACAAT -914 ATTATGATAAACATCTAAAAA -118 AGAGGGTTTAAAGTTTTTAATG -258 GATTTTTGGGTAAGGGAT -133 GGGTAGGGGTG -64 CCCCATACCCCAAA -18 GGGAAGTAGGGGTT -252 GGGTAGTTAGGGTT -253 GGGTAGTGAGGGGT -44 GGGAAGTAGGGGTT -18 GGGAAGTTAGGGGTT -292 GAAGTTTAGTGGATAGGGGT -292 GAAGTTTATGTATGTAGGAT -376 TATTTTTTTTTTATGGTGGTTTA -419 AATACCCAAATT -429 AATACCCAAATT -481 AGGAGGTTTAGGG -59 CCTAAACCCCAATTACCCCTC -134 GTAAAAAAATTAGAGTTTAGGGA -231 AGTAAAAAAATTAGAGTTTAGGGAA -232 AGTAAAAAAATTAGAGTTTAGGGAA -231 ACTAAAAAAAAAAAAAACCCCCTC -334 GTAAAAAAAAAAAAAA	-1661	TGGTTTTGAGGATTGTTAA
-1278 GGTTTTTATTTTTTAGTGAGAGATG -1156 GGGTTTTATATTTTTTAGTGAT -1119 AAAATAAACCTACACTCC -1010 CCTCAAACCCCAACT -1013 ATTATACAAACTCCAACAAAT -914 ATTATGATACAACATCATAAAA -914 ATTATGATACAACTCAAACAAAT -914 ATTATGATACAACACATCAAACAAT -914 ATTATGATAGAGTGTTG -914 ATTATGATTATAGAGATGTTAAA -914 ATTATGATTATGATGTTTG -914 ATTATGATACAACTCCAACAAT -914 ATTATGATTATAGAGAGAGTTGTG -914 AGAGGGTTTAAAGTTATGGGT -914 GGGTAGAGGAGGAGT -133 GGGTAGAGGAGGAGT -134 GGGTAGAGGGGT -252 GGGTATTTTGGGGTT -252 GGGTATTTTGGGGGTT -252 GGAGATTTGTTGGGGGT -252 GGAGTTTATAGTGATGGGTT -254 ATTACCTATTCCTACAAAT -429 ATAACTCATTCCTACAAATT -429 ATAACTCATTGCTACGAATT -481 GGGAAGTTGGTTTGGGG -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTAGGGA <td< td=""><td>-1337</td><td>AGATTGGGTAGGGTT</td></td<>	-1337	AGATTGGGTAGGGTT
-1156 GGGTTTTATAATTTTTTAGTGAT -1119 AAAATAAACCTAACTACATCC -1071 CCTCAAACCCCAACT -1071 CCTCAAACCCCAACAT -1013 ATTATACAAACTCACAACAAT -914 ATTATACAAACTCAACAAAT -914 ATTATGTTTAATGATGATGTTTG -817 CCTCAATCAAACAATCTTAAAA -718 ACAGGGTTAGAGAGTGT -64 CCCAATACCCCCAAA -18 GGGTAAGGGAGT -277 GGGTATTATGTGGTT -282 GAAGTTTATGGGGT -292 GAAGTTAATGTAAGGAAT -376 TTATTTTTTTGGGTTAATGTGTGTTTA -429 AATAACTCTATTTTGGGGTT -481 ACAGATTTTTGTTAGGG -64 ELOVL 5 -59 CCTAAACCCAAATTAACCCTC -134 GTAAAAAATTAGGGTTTAGGG -231 ACTAAAAAAATTAGAGGTTTAGGGAA -232 ACTAAAAAAATTAGAGGGAGAG -233 CCTAAACCCAATTAATTTTTTTAGGGG -244 ACAGATTTTGTTAGGG -259 CCTAAACCCAAATTAACCCCTC -234 ACTAAAAAAATTAGAGGTTTAGGG -235 ACTAAAAAAATTAGAGGTTTAGGAA -2	-1278	GGTTTTTATTTTTAAGTGAGATG
-1119 AAAATAAACCTAACTACATCC -1071 CCTCAAACCCCAACT -1013 ATTATACAAACTCCAACAAT -914 ATTATIGTITAATGATGTGTTTG -917 CCTCTAATCAAACATCTTAAAA -718 AGAGGGTTAAAGTTTTTAAT -258 GATTTTTTGGTA -133 GGGTAGAGGAGGTAT -64 CCCAATACCCCAAA -18 GGGGAGGGGT -64 CCCAATACCCCAAA -18 GGGGAGTAGGGGAGT -272 GGGTAGTTAGGGAT -2737 GGGTATTTGGAGGT -274 GGGTATTTAGTGAAGTAGAGT -275 GAGTTTTAGTGGGTT -276 GGGTATTTGGGGTT -277 GGGTATTTAGTGAAGTAGAGT -279 GAAGTTTAGTGAAGTAGAGT -279 GAAGTTTAGTGAAGTAGAGT -376 TTATTTTTGGGGTTTTA -429 AATAACTCTATTCCTAACAATT -429 GAGGAGTTTTAGTGGGG -6 ELOVL 5 -59 GTAAAAAATTGAGGTTAGGGA -231 GTAAAAAAATTAGAGGTTAGGGAGGGGGGGGGGGGGGG	-1156	GGGTTTTATAATTTTTTAGTGAT
-1071 CCTCAAACCCCAACT -1013 ATTATACAAACTCCACAAAT -914 ATTATIGITTAATGATGTGITTG -817 CCTCTAATCAAACAATCTTAAAA -718 AGAGGGTTAAAGTITITTAAT -258 GATTITTTGGGTAATGGTA -133 GGGTAGAGGAGGTG -64 CCCAATACCCCAAA -18 GGGAAGTTAGGTA -278 GGGTAGAGGAGGTG -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -279 GAGTTTATGTGAAGTATAGGGT -271 GGGTATTTGGGGTT -272 GAGTTTTATGTGAAGTAGAGT -274 GAGTTTTATGTGAAGTAGAGT -275 GAGTTTTATGTGAAGTAGAGT -276 TTATTTTTGGGGTT -277 GGGTATTTATGTGAAGTAGAGT -278 AATAACTCTATTCGTACAAATT -429 AATAACTCTATTCGTACAAATT -429 AGAGATTTTTGTTAGGG -6 ELOVL 5 -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGGTTAGGGA -231 ACTAAAAAAAATTGAGAGTTAGCACTCCATCA -232 <td>-1119</td> <td>ΑΑΑΑΤΑΑΑCCTAACTACATCC</td>	-1119	ΑΑΑΑΤΑΑΑCCTAACTACATCC
-1013 ATTATACAAACTCCAACAAAT -914 ATTATGTTTAATGATGTGTTTG -817 CCTCTAATCAAACAATCTTAAAA -718 AGAGGGTTTAAAGTTTTTAAT -258 GATTTTTTGGGTTAATGGTA -133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTAGGGAGTT -227 GGGTATTTTGGGGTT -237 GGGTATTTTGGAGTT -292 GAAGTTTAATGTAAGAT -376 TTATTTTTTTGAGGGT -411 AGAGATTTATTGTAAGTAAGAT -292 GAAGTTTATTGTAAGTAAGAT -376 TTATTTTTTTTATGGGG -411 AGAGATTTTGTTACCAAAATT -429 AATAACTCTATTCCTACAAATT -431 GGGAAGTTATGTTTTTTTTTTTTTTTTTTTTTTTTTTT	-1071	ССТСАААССССААСТ
-914 ATTATTGTTTAATGATGTGTTTG -817 CCTCTAATCAAACAATCTTAAAA -718 AGAGGTTTAAAGTTTTTAAT -258 GATTTTTTGGGTTAATGGTA -133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -257 GGGTATTTTGGGGTT -237 GGGTATTATGTAAGGAT -292 GAAGTTTATTGTAAGGAGT -376 TTATTTTTTTATGTGGGGTT -419 AATAACTCTATTCCTACAAATT -429 AATAACTCTATTCCTACAAATT -481 AGGAGTTTTGTTAGGG -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTAGGGAGA -231 AGTAAAAAAATTAGAGGTATAGGGAGA -236 ACTAAAAAAATTAGAGGAGAA	-1013	ΑΤΤΑΤΑCAAACTCCAACAAAT
-817 CCTCTAATCAAACAATCTTAAAA -718 AGAGGGTTTAAAGTTTTTAAT -258 GATTITTIGGGTAATGGTA -133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -227 GGGTATTTTGGGGTT -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAT -376 TTATTTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGAGATTTTGTTAGGG -59 CCTAAACCCAAATTAAGTGAGGAGGAG -134 GTAAAAAAATTAGAGGTAGGGAG -231 AGTAAAAAAAATTAGAGGTAGGGAGA -232 AATTAACCACTTCATTAATTATAGAGGAGAGA -233 AGTAAAAAAAATTAGAGTTAGGGAGAGGAGA	-914	ATTATTGTTTAATGATGTGTTTG
-718 AGAGGGTITAAAGTITITTAAT -258 GATTITTIGGGTAATGGTA -133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -227 CGGTATTITGGGGTT -237 GGGTATTATGGAGT -292 GAAGTTTAATGTAAGGAT -376 TTATTITTTAATGTGTGTTITTA -429 AATAACTCTATTCCTACAAATT -481 AGAGGTTTTAGGG -59 CCTAAACCCAAATTAGGGG -134 GTAAAAAATTAGGGGAGA -231 AGTATAAAAATTAGAGGAGAA -232 AATAACCCCAATTTAAGGGAGAA -336 CCTAAACCCAAATTAGGGGAGA -237 AGTATAAAAATTAGAGGAGAA	-817	ССТСТААТСАААСААТСТТАААА
-258 GATTTTTGGGTTAATGGTA -133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAAT -376 TTATTTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGGAATTTTGTTAGGG -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTAGGGA -231 AGTATATTATAGGGAGAGA -232 AATAAACTCAATTTATAGAGGAGA	-718	AGAGGGTTTAAAGTTTTTAAT
-133 GGGTAGAGGAGGTGT -64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -237 ELOVL2 -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAT -376 TTATTTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGAGATTTTAGGG -6 ELOVL 5 -59 CCTAAACCCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTTAGGG -231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAATCCACTTCATC -269 ACTAAAAAAATCCACTTCATC	-258	GATTTTTTGGGTTAATGGTA
-64 CCCAATACCCCCAAA -18 GGGAAGTTAGGGATT -18 ELOVL2 -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAT -376 TTATTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGAGATTTTGTTAGGG -6 ELOVL 5 -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTTAGGG -231 AGTAGTATAAATTTATAGAGGAGA -269 ACTAAAAAAAATCCACTTCATC	-133	GGGTAGAGGAGGTGT
-18 GGGAAGTTAGGGATT ELOVL2 -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAT -376 TTATTTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGGAGATTTTGGTG -59 CCTAAACCCAAATTAAGCGC -134 GTAAAAAAATTAGAGTTTAGGG -231 AGTAGATAAAAAATTCAAGGAGA -269 ACTAAAAAAAATCCACTTCATC 632 AATAACCAATTAACCAATTAACTATA	-64	СССААТАСССССААА
ELOVL2 -237 GGGTATTTTGGGGTT -292 GAAGTTTAATTGTAAGTAAGAT -376 TTATTTTTTAATGTGTGTTTTA -429 AATAACTCTATTCCTACAAATT -481 AGAGATTTTGTTAGGG -6 ELOVL 5 -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGGTTAGGG -231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAATCCACTTCATC	-18	GGGAAGTTAGGGATT
-237GGGTATTTTTGGGGTT-292GAAGTTTAATTGTAAGTAAGAT-376TTATTTTTTTAATGTGTGTTTTA-429AATAACTCTATTCCTACAAATT-481AGAGATTTTGTTAGGG-6ELOVL 5-59CCTAAACCCAAATTAACCCCTC-134GTAAAAAAATTAGAGTTTAGGG-231AGTATGATATAATTTATAGAGGAGA-269ACTAAAAAAATCCACTTCATC632AATTACAATTAACAAAAATTA		ELOVL2
-292GAAGTTTAATTGTAAGTAAGAT-376TTATTTTTTTAATGTGTGTTTTA-429AATAACTCTATTCCTACAAATT-481AGAGATTTTGTTTAGGG-6ELOVL 5-59CCTAAACCCAAATTAACCCCTC-134GTAAAAAAATTAGAGTTTAGGG-231AGTATGATATAATTTATAGAGGAGA-269ACTAAAAAAATCCACTTCATC	-237	GGGTATTTTTGGGGTT
-376TTATTITITTAATGTGTGTTTTA-429AATAACTCTATTCCTACAAATT-481AGAGATTTTGTTAGGG-6ELOVL 5-59CCTAAACCCAAATTAACCCCTC-134GTAAAAAAATTAGAGTTTAGGG-231AGTATGATATAATTTATAGAGGAGA-269ACTAAAAAAATCCACTTCATC633AATTACAATTAACAAAAATTA	-292	GAAGTTTAATTGTAAGTAAGAT
-429AATAACTCTATTCCTACAAATT-481AGAGATTTTGTTTAGGG-6ELOVL 5-59CCTAAACCCAAATTAACCCCTC-134GTAAAAAAATTAGAGTTTAGGG-231AGTATGATATAATTTATAGAGGAGA-269ACTAAAAAAATCCACTTCATC633AAATACCAATTAACCAATAAATAA	-376	TTATTTTTTTAATGTGTGTTTTA
-481 AGAGATTITTGTTAGGG -6 ELOVL 5 -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTTAGGG -231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAATCCACTTCATC 633 AAATTACCACTTUTTAACAAAAATTA	-429	ΑΑΤΑΑCTCTATTCCTACAAATT
-6 ELOVL 5 -59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTTAGGG -231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAATCCACTTCATC 633 AAATTACAATTTAACAAAAATTA	-481	AGAGATTTTTGTTTAGGG
-59 CCTAAACCCAAATTAACCCCTC -134 GTAAAAAAATTAGAGTTAGGG -231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAAATCCACTTCATC	-6	ELOVL 5
-134 GTAAAAAAATTAGAGTTTAGGG -231 AGTATGATATATATAGAGGAGA -269 ACTAAAAAAATCCACTTCATC 633 AAATTACAATTATAGAGAAAATAT	-59	ССТАААСССАААТТААССССТС
-231 AGTATGATATAATTTATAGAGGAGA -269 ACTAAAAAAAATCC 633 AAATTACAATTTTAACAAAAATAT	-134	GTAAAAAAATTAGAGTTTAGGG
-269 ACTAAAAAAAATCCACTTCATC	-231	AGTATGATATAATTTATAGAGGAGA
422 AAATTACAAATTTAACAAAAATAT	-269	ΑCTAAAAAAATCCACTTCATC
	-633	AAATTAGAATTTTTAAGAAAAATAT

TSS, transcription start site.

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Isolation of DNA and analysis of DNA methylation

Genomic DNA was isolated from 200 μ l PBMC or whole blood using the QIAmp DNA blood mini kit (Qiagen, 51106) according to the manufacturer's instructions. The level of methylation of individual CpG dinucleotides in the 5' regulatory regions of FADS 1 and 2, and ELOVL 2 and 5 was measured from -504 to -1037, from -18 to -1661, from -292 to -508 and from -6 to -686 bases, respectively, upstream from the transcription start site (Figure 1) using sodium bisulphite pyrosequencing essentially as described [10]. PCR primers and pyrosequencing probes are listed in Tables 2 and 3. Bisulphite conversion was carried out using the EZ DNA methylation kit (ZymoResearch, D5006). Modified DNA was amplified using KAPA2G Robust Hot Start Taq DNA polymerase (Anachem, KK5702). PCR products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd., 17-5113-01), washed, denatured and released into annealing buffer containing the sequencing primers. Pyrosequencing was carried out using CDT reagents (Qiagen, 972824) on a PSQ 96MA machine (Qiagen, 972824). Per cent methylation at each CpG locus was calculated using the Pyro Q CpG software (Biotage). The within assay coefficient of variation was less than 5% for all CpG loci that were measured and the limit of detection was 5% methylation.



Figure 2. Values are mean ± SEM methylation of individual CpG loci at baseline in the 5' regulatory regions of (A) FADS2, (B) FADS1, (C) ELOVL5 and (D) ELOVL2 in PBMCs from male (open bars) and female (closed bars) subjects in the Study 1 cohort. Numbers of subjects are listed in Table 1. Locations of individual CpG dinucleotides are relative to the transcription start site (TSS). Dotted horizontal line indicates the analytical limit of the assay. doi:10.1371/journal.pone.0109896.q002

Analysis of mRNA expression

mRNA was isolated from PBMCs using Tri Reagent (Sigma, T9429). Complementary DNA was prepared and amplified by real-time RT PCR using SYBR Green Jumpstart Ready Mix (Sigma, S4438) as described previously [14]. The level of mRNA expression was quantified using commercially prepared primer pairs for FADS1 Hs_FADS1_1_SG QuantiTect primer assay, FADS2 (Hs_FADS2_1_SG QuantiTect primer assay), ELOVL 2 (Hs_ELOVL2_1_SG QuantiTect primer assay) (all from Qiagen). The assay conditions were as described by the manufacturer and were validated in house before use. ELVOL5 was quantified using bespoke primer sets (5' to 3' TATGAAGATTATCCGTGTC; 3' to 5' TGGCACCAAAATAAGAGT) (Eurofins Genomis, http:// eurofinsgenomics.eu). Cycle parameters were 95°C for 2 minutes then 40 cycles of 95°C for 30 s, 55°C (cyclophilin, FADS1, FADS2, ELOVL2) or 60°C (ELOVL5) for 1 min and 72°C for 1 min. Samples were analysed in duplicate and the level of the individual transcripts was normalised to cyclophilin (Qiagen, Hs_PPIA_1_SG QuantiTect primer assay) by the standard curve method [15].

Statistical analysis

There are no previous data sets of methylation levels of the genes of interest on which to base calculations of sample size. Therefore, retrospective analysis of statistical power was carried out for exemplar levels of methylation of 95%, 47% and 17% in FADS2. For these levels of methylation, 7 subjects per group would provide a statistical power of 80% to detect a difference of 10% with a probability of P<0.05. Male and female subjects were analyzed separately. The primary statistical analyses were as follows. Statistical comparisons of methylation level at baseline between male and female subjects were by Student's unpaired t test. Comparison of end of treatment values with baseline values was by Student's paired t test. The effect of treatment was determined by ANCOVA with supplement as a fixed factor and baseline methylation, age, BMI, proportion of lymphocytes and neutrophils as covariates with Bonferroni's post hoc correction. The relationship between the level of methylation at individual CpG loci and that of the mRNA expression of the corresponding transcript was by Pearson's correlation analysis.



Figure 3. Values are mean \pm SEM difference in methylation from baseline of individual CpG loci in the 5' regulatory regions of (A, B) FADS2, (C, D) FADS1, (E, F) ELOVL5 and (G, H) ELOVL2 in PBMCs from male (A, C, E, G) and female (B, D, F, H) subjects in the Study 1 cohort who received n-3 LCPUFA (open bars) or OO (closed bars) supplements. Numbers of subjects are listed in Table 1. Locations of individual CpG dinucleotides are relative to the transcription start site (TSS). *Means that were significantly different by Student's paired t test between baseline and end of intervention samples are indicated by ^aP<0.05, ^bP<0.01, ^cP<0.001, ^dP<0.0001. doi:10.1371/journal.pone.0109896.q003

Results

Subjects

There were no significant differences by one-way ANOVA in the characteristics of the subjects at baseline between the dietary supplementation groups in the Study 1 cohort (Table 1). However, the subjects in the Study 2 cohort were significantly younger (P< 0.001) and had a lower BMI (P<0.001) than the subjects in the Study 1 cohort (Table 1). There were no significant differences in subject characteristics between males and females in either cohort.

Methylation at baseline in the Study 1 cohort

Since the limit of detection was 5%, CpG loci that exhibited methylation of 5% or less were regarded as essentially unmethylated. This is consistent with previous reports [16]. The level of methylation of FADS2, FADS1 and ELOVL5 tended to be related inversely to distance from the transcription start site (TSS) (Figure 2 A-C). While this transition was gradual for FADS2, there appeared to be a sharp demarcation between a highly methylated (>70%) region distal to the TSS and the proximal region which was essentially unmethylated region in FADS1 and ELOVL5 which is consistent with the presence of CpG islands within the proximal promoters of FADS1 and ELOVL5 (Figure 1). Methylation of individual CpG loci in the 5' regulatory region of ELOVL2 appeared to be essentially uniform with methylation levels between approximately 10% to 20%, with the exception of a CpG located at -429 bp from the TSS (CpG -429) which had a markedly greater level of methylation, approximately 80%, compared to the other CpG loci that were measured in this gene (Figure 2D).

Effect of dietary supplementation with n-3 LCPUFA or OO on the methylation of individual CpG loci in the Study 1 cohort

Both OO and n-3 LCPUFA induced significant changes compared to baseline in the level of methylation of individual CpG loci in specific genes involved in PUFA metabolism in PBMCs in the Study 1 cohort that were contingent on sex. In males, n-3 LCPUFA increased methylation at CpG -806 (12.5%) in FADS2 compared to baseline, but decreased methylation at CpG-775 (11.8%) (Figure 3A). Supplementation with OO increased the level of methylation compared to baseline at both of these loci by 13% and 24.0%, respectively (Figure 3A). In females, n-3 LCPUFA supplementation induced increased methvlation of FADS2 at CpGs -1071 (8.0%), -975 (6.2%), -871 (4.6%) and at CpG-775 (8.9%) compared to baseline (Figure 3B). Supplementation with OO increased methylation at CpGs -1119 (6.3%), -1101 (14.6%), -871 (13.1%), -869 (16.5%), -855 (13.3%), -817 (17.5%), -806 (13.9%) and CpG -775 (8.9%) compared to baseline (Figure 3B).

In males, supplementation with n-3 LCPUFA increased the level of methylation of CpG -686 (10.1%) compared to baseline in ELOVL5, but did not alter significantly the level of methylation of the other CpGs that were measured (Figure 2E). There was no significant effect of supplementation with OO on the methylation of CpG loci in ELOVL5 (Figure 2E). In females, n-3 LCPUFA

supplementation increased the level of methylation of CpG -686 (3.1%) and CpG -269 (2.1%) in ELOVL5 compared to baseline (Figure 3F). Supplementation with OO decreased methylation of CpG -686 (3.1%), but increased methylation of CpG -269 (7.6%) and CpG -259 (4.6%) compared to baseline (Figure 2F). There was no significant effect of supplementation with either n-3 LCPUFA or OO on the level of methylation of any of the CpGs measured in FADS1 or in ELOVL2 in males or females (Figure 3C, D, G, H).

In males, the type of oil used in the dietary supplement induced differential methylation of CpG -775 (22%) in FADS2 (Table 4) and of CpG -686 (11%) in ELOVL5 (Table 5). In females, supplementation induced differential methylation of CpGs -1071 (8.5%), -975 (4.1%), -871 (8.2%), -869 (10.7%), -855 (10.0%), -817 (22.1%) and -775 (7.4%) in FADS2 (Table 4). The type of dietary oil supplement induced differential methylation at CpG -686 in males (11%) and in females (6.5%) in ELOVL5 (Table 5). There were no significant differences between the effects of the dietary supplements on the methylation of other CpG loci in FADS2 or ELOVL5, nor in the level of methylation of any other CpG loci measured in FADS1 or ELOVL 2 in males or females (Tables 4 and 5).

Effect of dietary supplementation with n-3 LCPUFA on the methylation of individual CpG loci in the Study 2 cohort

We investigated whether the changes in methylation induced by supplementation with n-3 LCPUFA in the Study 1 cohort could be replicated in a second cohort of health individuals. For FADS2, n-3 LCPUFA supplementation in the Study 2 cohort also induced an increase in methylation at CpGs -803 and -775 in males and in CpGs -1071, -975 and -775 in females (Figure 4A) to those induced in the UWA cohort (Figure 2 A, E). However, increased methylation at CpG -871 in females in the UWA cohort was not replicated in the Study 2 cohort (Figure 4A). For ELOVL5, the level of methylation of CpG -686 was increased compared to baseline in both the Study 1 and Study 2 cohorts (Figure 4B). However, the level of methylation of CpG -269, which was increased compared to baseline in females who received the n-3 LCPUFA supplement in the Study 1 cohort, was unchanged in females in the Study 2 cohort. CpGs in FADS2 or ELOVL5 that were measured in the same amplicons as those that showed altered methylation, but which did not exhibit a change in methylation following n-3 LCPUFA supplementation in the Study 1 cohort, also did not show altered methylation in the Study 2 cohort (Figure 4).

The relationship between the methylation of individual CpG loci and the mRNA level of the corresponding transcripts

The level of methylation of specific CpG loci in FADS2 and ELOVL5, but not FADS1 nor ELOVL2, in the Study 1 cohort were associated significantly the level of the mRNA transcript in samples collected at the end of the study irrespective of subject sex or dietary supplement. There were no significant differences in FADS1 or ELOVL2 mRNA expression between baseline and end of study samples (Figure 5 A, C). However, the mRNA expression of FADS2 was reduced significantly in all dietary groups, while

Table 4. Effect of supplementation with n-3 LCPUFA or olive oil on DNA methylation of FADS2 and FADS1 in the Study 1 cohort.

	Methylation of in	idividual CpG loci	(%)					
	Male				Female			
CpG	n-3 LCPUFA	0	Adj n-3 LCPUFA	Adj OO	n-3 LCPUFA	8	Adj n-3 LCPUFA	Adj OO
	FADS2							
-1661	95.0±1.6	93.6±1.2	95.1±0.5	93.4±0.6	92.4±4.4	94.0±1.5	93.7±1.5	93.7±1.2
-1665	88.7±1.1	88.7±1.8	88.8±0.4	88.7±0.5	84.6±2.2	89.5±1.3	84.5±2.3	89.6±2.2
-1337	83.7 ± 3.5	81.6±3.2	82.8±0.8	81.3±0.8*	79.6±3.7	7.77±5.2	79.7±2.1	77.2±2.2
-1156	61.9±8.8	61.3±5.3	61.9±3.0	61.3±2.6	61.7±7.0	58.4 ± 5.8	61.1±2.4	59.8±2.3
-1119	22.8±5.2	22.8±5.8	22.7±1.9	21.9±2.1	25.8±6.3	28.5±7.8	25.8±2.1	29.9±2.3
-1112	41.7±5.2	34.0±5.0	41.7±2.4	33.9±2.0	31.3±8.9	40.9±7.3	39.2±3.4	41.9±3.8
-1101	34.9±8.1	35.9±8.0	34.6±2.3	36.3±2.5	35.8±4.7	41.2±2.9	37.5±3.4	40.9±7.6
-1071	44.6±3.2	45.0±4.4	44.7±1.9	44.8±2.6	53.4±7.7	43.4±5.2	52.6±1.3	44.1±1.3*
-1067	46.7±4.7	49.7±8.1	46.5±2.0	49.6±2.7	47.2±6.3	46.3±7.3	48.3±4.0	47.6±3.6
-1056	36.9±7.3	39.0±8.4	40.14.4	39.1±3.5	35.7±5.9	37.4±7.1	35.6±4.2	37.1±3.4
-1013	46.1 ± 3.0	39.4±8.8	46.2 ± 3.0	39.4±2.6	44.8±4.5	43.7±4.7	44.9±1.8	44.1±1.8
-975	52.7±5.1	48.3±10.1	52.3 ± 2.4	50.5 ± 2.6	60.8±8.3	48.1±4.7	57.3±1.4	53.2±1.5*
-914	19.6±6.3	20.8 ± 5.2	19.8±1.3	20.6±1.6	21.7±5.8	18.3±5.1	21.9±2.8	19.8±2.9
-871	25.4±5.8	30.3±4.4	27.9±1.6	29.3±1.7	30.6±7.2	33.1±8.3	27.2±3.0	35.4±2.8*
-869	21.9 ± 5.4	24.0±6.4	22.6±2.7	23.6±3.6	27.4±5.9	33.1±4.0	23.7±1.5	34.4±1.9**
-855	27.6±4.9	22.1±9.8	29.1±2.8	20.8±3.9	29.2±4.7	35.6±4.4	28.7±1.0	38.7±1.4**
-817	24.1 ± 5.4	21.2±4.9	23.7±4.3	22.4±3.6	17.7±11.8	31.5±5.6	14.8±4.9	36.9±5.7**
-806	50.0 ± 2.3	50.8 ± 2.0	50.0 ± 0.9	50.9 ± 1.0	38.0±9.2	37.7±5.7	37.8±1.5	$51.6\pm4.0^{**}$
-775	23.6±7.8	45.8±4.3	23.6±7.7	45.8±4.36**	47.0±3.5	33.5±2.1	44.8±4.3	37.4±2.2**
-64	1.6 ± 0.9	2.1 ± 1.2	1.6±0.3	2.1 ± 0.4	1.7±0.9	1.9 ± 0.3	1.6±0.3	1.9±0.2
-50	3.8 ± 0.9	5.7±3.4	3.7±0.6	5.7 ± 0.8	3.6±0.5	3.5±0.9	3.6±0.3	3.5±0.3
-18	4.1±0.7	4.2 ± 0.5	4.1±0.3	4.1 ± 0.4	4.1±0.7	4.4±0.9	4.2±0.4	4.3±0.4
	FADS1							
-1037	76.3±4.3	78.2±4.3	77.9±2.8	76.6±2.2	74.8±2.0	75.4±5.2	72.7±2.0	69.7±1.9
-830	83.3 ± 2.8	82.9±1.9	83.4±0.7	82.7±0.8	84.1±2.5	85.2±3.8	85.2±1.2	84.1±1.9
-586	26.5 ± 5.7	28.1±5.1	27.2±2.0	27.8±1.9	23.0±5.1	23.3±3.6	23.7±1.6	22.7±2.3
-528	1.9±1.0	3.7±1.8	2.9±0.7	3.7±0.6	3.0±1.0	2.6±0.8	2.6±0.3	2.6±0.4
-512	1.0±1.2	2.2±1.7	1.8 ± 0.6	1.8±0.3	2.8±0.5	2.8±0.7	2.9±0.2	2.7±0.3
-507	2.9±1.6	3.6±1.1	3.0±0.6	3.7±0.6	3.3±0.7	2.2 ± 1.5	3.4±0.3	2.6±1.0
-504	2.0±2.1	2.6±1.2	2.2±0.5	2.6 ± 0.5	3.0±0.9	2.46±1.4	3.1±0.3	$2.8\pm .0.5$
Values are I indicated in baseline, ag Individual C	mean or adjusted (Adj) r n Table 1. Comparisons b Je, BMI, and proportion c - pG dinucleotides are in	mean ±SEM methylic petween the level of of lymphocytes and i dicated by their pos.	ation of individual CpG loci at methylation of individual CpG neutrophils in blood, with Bonf sition relative to the transcriptic	the end of the dietary int loci between supplement ferroni's <i>post hoc</i> correctio on start site (bp).	ervention period in indivi ation groups at the end o in. Adjusted means that w	duals who received eith f the intervention were ere differed significantl	rer the n-3 LCPUFA or OO suppler by ANCOVA. Means were adjustec y are indicated by *P<0.05, **P<0	nents. Numbers of subjects are I for the level of methylation at .01, ***P<0.001, ****P<0.0001.
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Male				Lallar			
 n-3 LCPUFA	00	Adj n-3 LCPUFA	Adj OO	n-3 LCPUFA	00	Adj n-3 LCPUFA	Adj OO
ELOVL 5							
83.4±3.4	76.4±2.5	85.0±1.1	74.0±2.8*	83.1±3.1	75.2±4.7	82.4±1.2	75.9±1.1**
77.2±8.9	74.6±3.2	77.4±3.2	78.2±5.5	72.4±2.3	72.1±4.2	72.5±2.3	72.1±4.2
5.6±1.2	5.1 ± 1.8	5.8 ± 0.6	4.3±0.6	9.8±1.2	4.8±1.5	4.7±0.7	5.9 ± 1.3
7.6±1.8	7.5±2.2	8.9±5.4	8.1±5.1	7.1±1.7	6.8±2.7	5.6±3.1	7.3±2.9
5.3 ± 0.8	5.1 ± 0.7	5.3 ± 0.3	4.9 ± 0.3	4.5±1.9	6.6 ± 0.8	5.1 ± 0.9	8.2±1.3
2.0 ± 0.5	2.1 ± 0.6	3.5±1.4	2.3 ± 0.2	2.9±0.8	2.6 ± 0.5	2.5 ± 0.3	3.0 ± 0.4
9.8±2.6	8.2 ± 1.1	8.0±0.5	9.4±0.7	8.1±1.0	9.9±2.1	9.3±0.8	8.1±0.9
13.3 ± 3.3	12.4±0.7	13.5±1.0	12.5 ± 1.5	14.5±2.4	12.7±1.2	14.1 ± 0.8	12.7 ± 0.9
2.8±1.5	3.5±1.0	2.5 ± 0.4	3.4±0.6	3.9±0.9	3.3±1.5	3.8 ± 0.4	2.9±0.5
6.6±2.0	6.3±1.9	6.1 ± 0.7	5.9±1.1	7.7±2.8	6.9±1.3	7.9±1.0	6.6 ± 1.3
3.6±0.9	3.7±2.0	3.6±0.4	3.8 ± 0.5	3.7±1.5	4.6 ± 1.2	3.7±0.5	4.6 ± 0.5
1.5 ± 0.6	2.1 ± 0.5	1.5±0.5	11.9 ± 6.2	1.8 ± 0.3	1.9 ± 0.4	1.8±0.1	2.4 ± 0.3
ELOVL 2							
18.4 ± 3.2	16.8±7.3	19.4±2.3	16.8 ± 2.1	17.3±6.4	20.6 ± 5.5	15.7±2.2	21.9±2.3
24.0 ± 5.7	22.3 ± 8.5	23.8±2.6	22.5 ± 2.6	24.8±6.4	22.1 ± 4.3	24.9±2.6	22.0±2.6
21.4±4.5	22.4±5.3	21.8±2.4	22.0 ± 2.0	24.1±6.2	23.8±6.2	23.8±2.3	24.12.1
16.3±4.3	15.1 ± 3.1	16.2±1.3	15.1 ± 1.3	14.8±3.6	16.2 ± 4.2	15.0±1.5	16.5 ± 1.3
77.5±6.9	74.4±4.2	78.0±2.0	75.1 ± 2.4	76.4±4.1	71.1±4.9	76.5±2.2	71.9±2.5
13.6±4.8	15.6±3.5	14.1±1.5	15.8±1.7	16.9±3.6	16.8±3.4	16.3±1.6	15.1 ± 1.8
20.1 ± 5.0	22.1 ± 3.7	20.0±1.2	21.9±1.4	19.3±2.7	21.5 ± 2.3	18.8±1.2	21.1±1.3
8.6±1.3	7.9±1.7	8.1 ± 0.4	8.7±0.5	8.6±2.7	9.7±1.4	8.8 ± 0.9	9.5 ± 0.9
14.1±1.9	12.6±4.9	14.1±1.1	13.1±1.4	12.1±6.7	14.9±2.6	10.8±1.7	14.7±1.7
8.9±2.9	11.0±1.1	8.9 ± 0.8	11.8±1.4	10.9±2.5	11.7±1.7	11.4 ± 0.8	11.8 ± 0.9
9.9±4.3	9.8±1.6	9.8±0.8	11.2 ± 0.8	10.2±1.5	10.3±2.7	10.0±0.8	10.9 ± 0.9

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Figure 4. Values are mean \pm SEM difference in the methylation of individual CpG loci from baseline in the 5' regulatory regions of (A) FADS2 and (B) ELOVL5 in PBMCs from male (open bars) and female (closed bars) in the Study 1 cohort who consumed a n-3 LCPUFA supplement. Numbers of subjects are listed in Table 1. Locations of individual CpG dinucleotides are relative to the transcription start site (TSS). *Means that were significantly different (P<0.05) by Student's paired t test between baseline and end of intervention samples. doi:10.1371/journal.pone.0109896.g004

ELOVL5 expression was reduced in females who received the OO supplement and in males who received with the OO or n-3 LCPUFA supplement at the end of the study compared to baseline (Figure 5 B, D). The CpGs at -119, -871, -869, -806 and -775, which showed altered methylation in response to dietary supplementation, were associated negatively with the level of the

FADS2 transcript (Table 6). The CpGs -686 and -259 in ELOVL5, which showed altered methylation in response to dietary supplementation, were associated negatively with the level of the ELOVL5 transcript (Table 6). These associations are illustrated for FADS2 CpG -775 and ELOVL 5 CpG -686 in Figure 6.



Figure 5. Change in relative mRNA expression of (A) FADS1, (B) FADS2, (C) ELOVL2 and (D) ELOVL5 in peripheral blood mononuclear cells from Study 1. *Means at the end of the end of the study that were significantly different (P<0.05) compared to baseline assessed by Student's paired t test. Combined data refers to the overall change in mRNA expression irrespective of sex or supplement (these data were used to test the statistical association with the methylation status of the respective genes). doi:10.1371/journal.pone.0109896.g005

Discussion

The findings of this study show for the first time that modest dietary supplementation with either n-9 monounsaturated fatty acids or n-3 LCPUFA induces altered DNA methylation in specific genes involved in LCPUFA metabolism in leukocytes from adults that differed between the type of supplement and between sexes.

The methylation profile of CpGs within the 5' regulatory region of the four key genes involved in LCPUFA biosynthesis has not been reported previously in humans. FADS2 was characterised by a gradual decline in the level of methylation of individual CpG loci 90% to 20% with decreasing distance from the transcription start site. Previous reports in rodents have shown that the level of methylation of the Fads2 promoter in mice was less than 10% [8,17], while in rats the level of methylation was between 30% and 90%, increasing with distance from the transcription start site [10,11]. This implies that the epigenetic regulation of this gene may be more similar in humans to rats, than to mice, which may have implications for understanding the epigenetic regulation of FADS2 in these species. However, since FADS2 appears to have multiple TSS (http://www.ensembl.org/Homo_sapiens/Gene/Summary? db=core;g=ENSG00000134824;r=11:61560452-61634826), detailed promoter mapping in the different species would be necessary to establish differential species-specific regulation of this gene. The level of methylation of some CpG loci in the 5' regulatory region of FADS2 has been reported previously in HepG2 cells [18]. Despite prolonged passage of this hepatocellular carcinoma cell line in culture, the methylation levels of the CpG loci that were measured were comparable to those reported here, with the exception of CpG -1661, which had approximately 30% lower methylation in HepG2 cells [18]. Whether this difference in methylation reflects an effect of prolonged adaptation to in vitro culture or differences in cell type between leukocytes and hepatocellular carcinoma cells cannot be determined from these findings. However, together these observations suggest that the epigenetic regulation of the FADS2 gene may be similar in the two distinct cell types and that HepG2 cells may be a suitable model of the epigenetic regulation of FADS2 in humans.

FADS1 and ELOVL5 showed a marked demarcation between a highly methylated region distal to the transcription start site and an essentially unmethylated proximal region. ELOVL2 showed a similar level of methylation, about 20% across the region that was measured with the exception of CpG -429 that showed markedly higher methylation. However, the role of this CpG locus in the regulation of ELOVL2 and the effect of differential methylation of this site on the transcription of the gene is not known.

There is substantial evidence that women have higher DHA status [19] and capacity for DHA synthesis [20,21] than men. This sex difference has also been shown in rodents to be accompanied by differential mRNA expression of Fads2 and Fads1 in the liver [22,23]. In the present study, we found no differences between men and women in the methylation status of individual CpG loci in any of the gene regions that were investigated. This suggests that differential methylation of FADS1 or 2, or of ELOVL 2 or 5 may not contribute significantly to the sex difference in DHA status. Previous studies have shown that supplementation of pregnant rodents with different types and amounts of dietary fats induced hypermethylation of specific CpG loci in the Fads2, but not Fads1, promoter in the liver and aortae of the offspring [10,11]. The nature of such changes differed according to the type and amount of fat in the maternal diet. Similarly, feeding adult rats a diet enriched in fish oil induced hypermethylation of specific CpG loci in the Fads2 promoter which was reversed by withdrawal of the high n-3 LCPUFA diet [10]. Feeding adult male mice a high fat diet induced hypermethylation of specific CpG loci in stearoyl-CoA desaturase [24]. Treatment of cells in culture with EPA has also been shown to induce altered methylation of sCCAAT/ enhancer binding protein- β [25]. To our knowledge there have been relatively few studies of the effect of dietary fat on DNA methylation in humans. Consuming a high fat diet for 5 days induced altered methylation of 7,909 CpG loci in 658 genes in

FADS2										
		FADS1			ELOVL 5			ELOVL 2		
CpG	٩	CpG	-	٩	CpG	-	٩	CpG	-	٩
-1661C	.05	-1037	-0.06		-686	-0.32	0.00	-508	0.3	
-1665 -C	.06	-830	-0.5		-633	0.04		-501	-0.44	
-1337 0.0	33	-586	60.0		-269	-0.11		-491	0.20	
-1156 0.	21	-528	-0.1		-266	-0.15		-481	0.1	
-1119	.57 0.009	-512	-0.24		-259	-0.18	0.038	-429	-0.25	
-1112 0.	15	-507	0.05		-231	-0.22		-350	-0.08	
-1101 0.	8	-504	-0.05		-185	-0.1		-344	0.05	
-1071 -C	:12				-180	-0.37		-335	0.24	
-1067 0.	1				-153	-0.1		-319	0.08	
-1056 0.	17				-134	0.0		-304	0.29	
-1013 0.	14				-14	-0.07		-292	-0.12	
-975 0.	11				-0	-0.08				
-914 0.0	10									
-871 -C	.17 0.047									
-869	.31 0.017									
-855 0.0	38									
-817 0.	12									
-806 -C	.52 0.007									
-775 -0	.21 0.043									
-64C	.02									
-500	.06									
-18 0.0	12									

Supplementation with Dietary Oils and DNA Methylation

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Figure 6. Change in relative mRNA expression compared to change in the methylation status of (A) FADS2 CpG -775 and (B) ELOVL5 CpG -686 irrespective of subject sex or supplementation group in peripheral blood mononuclear cells from Study 1. The corresponding correlation efficients are shown in Table 6. doi:10.1371/journal.pone.0109896.g006

skeletal muscle from young men [26]. Feeding 400 mg DHA per day to pregnant women from mid gestation to term induced a small increase (about 1%) in the methylation status of LINE-1 sequences in leukocytes from umbilical cord blood [27]. However, the nature of the latter study design may limit the interpretation of these findings [28]. The current findings show that consuming supplements enriched in n-9 monounsaturated fatty acids or n-3 LCPUFA induces differential changes in the methylation of individual CpG loci in two out of the four genes that were investigated. Such differential effects are consistent with previous findings in rodents [11]. Furthermore, the pattern of induced changes in DNA methylation differed between men and women such that in terms of the number of CpG loci that were affected, the effect of the dietary supplements was greater in women than in men in both FADS2 and ELOVL5.

The effects of supplementation with n-3 LCPUFA that were observed in the patients with chronic kidney disease were fairly well replicated in the cohort of healthy individuals in terms of the number and location of the CpGs that were changed in both FADS2 and ELOVL5, and the direction of the effect on these genes. There were two exceptions, CpG -871 in FADS2 and CpG -269 in ELOVL5 showed increased methylation (approximately 5%) in women in the Study 1 cohort who consumed the n-3 LCPUFA supplement, but were unchanged in the Study 2 cohort. However, overall these findings suggest that effect of supplementation with n-3 LCPUFA on DNA methylation of FADS2 and ELOVL5 was not affected significantly by differences in the fatty acid composition of the n-3 LCPUFA preparations, or the age, BMI, health status or geographical location of the subjects. Since the changes induced in the methylation of FADS2 and ELOVL5 were similar after either 8 or 12 weeks supplementation, these findings also suggest that the effects of increased intakes of n-3 LCPUFA had reached a maximum response by 2 months. CpGs in FADS2 and ELOVL that were proximal to those that showed altered methylation but were unaffected by the dietary supplement in the Study 1 cohort, were also unaffected in the Study 2 cohort. This confirms the specificity of the effects of the different fatty acid supplements.

The methylation status of specific CpG loci in Fads2 has been shown previously in rats to directly influence the level of transcription [11]. We investigated whether the level of methylation of individual CpG loci in the four genes of interest was associated with the level of the corresponding transcripts. We found that the methylation status of four CpG loci in FADS2 and one CpG loci in ELOV5, which showed induced changes in methylation following dietary supplementation, were negatively associated with the level of the FADS2 and ELOVL5 transcripts, respectively. Although this does not provide a direct functional assessment of the effect of altered methylation at these loci on the level of transcription, such associations are consistent with increasing DNA methylation inducing a lower level of transcription. Increased total dietary fat intake [6,7], or supplementation with olive oil [29] or n-3 LCPUFA [30] has been shown to decrease the conversion of 18 carbon essential fatty acids to their longer chain metabolites. The present findings suggest that increased intakes of n-3 LCPUFA or olive oil may reduce PUFA biosynthesis via changes in the epigenetic regulation of FADS2

and ELOVL5 that affect gene transcription, possibly in addition to any effect of product inhibition. Since olive oil is not a pure preparation of fatty acids, it cannot be assumed that the changes in DNA methylation were due to the lipid content of the oil since other biologically active compounds are present such as polyphenols [31]. Because D6d is the rate-limiting enzyme in PUFA biosynthesis, lower transcription of FADS2 would tend to downregulate the activity of the whole pathway. However, lower transcription of ELOVL5 may reduce conversion of dihomo- γ linolenic acid to archidonic acid and EPA to DPAn-3. If so, one possible consequence could be to increase the synthesis of substrates for the synthesis of prostaglandin (PG) E₁ and PGE3, relative to PGE₂.

The nature of this study design has some limitations. Although retrospective analysis suggested that the study was adequately powered to detect differences of 10%, changes in methylation of less than 10% need to be considered cautiously. Replication of the study in a larger prospective cohort of healthy individuals, preferably with a crossover rather than parallel design, would be desirable to substantiate the current findings. It would also be desirable to test the dietary supplements at more than one dose. Because cell differentiation involves differential changes in the methylation of individual genes, it is possible that variations in cell populations may have contributed to the variation in the level of methylation of individual CpG loci, although differences in the proportions of individual cell populations were taken into account in the analysis of the samples from the Study 1 cohort. Furthermore, the similarities in the findings from the Study 2 cohort, which were based on DNA isolated from whole blood without correction for leukocyte sub-types, to those from the Study 1 cohort supports the suggestion that variation in cell populations does not significantly affect the methylation of these genes. Finally, although there were significant associations between the methylation status of individual CpG loci and the level of the corresponding mRNA transcripts, the effect of variation in DNA methylation of these genes on their transcription and on PUFA biosynthesis remain to be demonstrated directly.

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Overall, the findings of this study show that dietary supplementation of adult humans with modest amounts of n-3LCPUFA or olive oil can induce selective changes in the methylation status of individual CpG loci in specific genes, which is contingent on the sex of the subject and the nature of the supplement. Such findings may have implications for understanding the mechanisms the underlie the health benefits associated with higher consumption of fish oil [32,33] or olive oil [34]. Furthermore, altered DNA methylation has been implicated as a causal process in a number of non-communicable diseases [4,5]. One challenge in the design of therapeutic strategies to ameliorate or reverse altered patterns of DNA methylation in disease states is targeting the intervention to specific CpG loci [35]. One possible implication of the current findings is that supplementation with specific fatty acids or combinations of fatty acids may provide a means of delivering health benefits by inducing selective modification of the methylation status of specific CpG loci.

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Author Contributions

Conceived and designed the experiments: GCB KAL TAM PCC. Performed the experiments: SPH RH. Analyzed the data: GCB. Contributed reagents/materials/analysis tools: TAM PCC. Contributed to the writing of the manuscript: GCB KAL PCC TAM LJB RC-H.

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