Enrichment of n-3 containing ether phospholipids in plasma after 30 days of krill oil compared with fish oil supplementation

Received: 5 November 2021 | Revised: 15 December 2021 | Accepted: 15 December 2021

DOI: 10.1002/lipd.12335

ORIGINAL ARTICLE

AOCS WILEY

Enrichment of n-3 containing ether phospholipids in plasma after 30 days of krill oil compared with fish oil supplementation

Hyunsin (Hedy) Sung[1] | **Andrew J. Sinclair**[2] | **Xiao Q Su**[1]

[1]Institute for Health and Sport, Victoria University, Melbourne, Victoria, Australia

[2]Department of Nutrition, Dietetics and Food, Monash University, Notting Hill, Victoria, Australia

Correspondence
Xiao Q Su, Institute for Health and Sport, Victoria University, Melbourne, Vic, Australia.
Email: xiao.su@vu.edu.au

Andrew J. Sinclair, Department of Nutrition, Dietetics and Food, Monash University, Notting Hill, Vic, Australia.
Email: andrew.sinclair@monash.edu

Funding information
Victoria University

Abstract

There are conflicting findings over the bioavailability of long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) from krill oil (KO) compared with fish oil (FO) in short- and long-term studies. The aim of this study was to compare the effects of KO versus FO on the enrichment of molecular species of plasma phospholipids in young women following a 30-day consumption of the n-3 oils. Eleven healthy women aged 18–45 years consumed seven capsules of KO per day (containing a total of 1.27 g n-3 PUFA) or five capsules of FO per day (total of 1.44 g n-3 PUFA) for 30 days in a randomized crossover study, separated by at least a 30-day washout period. Fasting blood samples were collected at day zero (baseline), day 15 and day 30 and analyzed by HPLC-MS/MS for molecular species of phospholipids. Supplementation increased n-3 PUFA in main phospholipids classes in both groups. After 30 days of supplementation, 35 out of 70 molecular species containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPAn-3) had a significantly greater concentration in KO group compared with the FO treated group. The majority (89%) of the differentiated molecular species were choline and ethanolamine ether-phospholipids. These data reveal that analysis of plasma phospholipids following 30 days of consumption of KO (a marine oil rich in phospholipids, including ether phospholipids) resulted in an enrichment of n-3 PUFA in molecular species of ether-phospholipids compared with FO (a triacylglycerol-rich marine oil).

KEYWORDS
cross-over study, DHA, EPA, ether phospholipids, fish oil, krill oil, plasmalogens, supplementation

INTRODUCTION

Krill oil (KO) extracted from crustaceans (*Euphausia superba*), a shrimp-like marine zooplankton living in the Antarctic, has been recognized as an important source of long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) in the last decade (Tou et al., 2007). KO is a rich source of long-chain n-3 PUFA, found in both the sn-1 and sn-2 positions of phospholipids (mainly phosphatidylcholine (PtdCho), triacylglycerols (TAG), and in free

Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; $CHCl_3$:MeOH, chloroform:methanol; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; FO, fish oil; HPLC ESI-MS/MS, high-performance liquid chromatography electrospray ionization-tandem mass spectrometry; KO, krill oil; PakCho, alkyl-phosphatidylcholine; PlsCho, phosphatidylcholine plasmalogen; PlsEtn, phosphatidylethanolamine plasmalogen; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; SEM, standard error mean; TAG, triacylglycerols.

fatty acids (Winther et al., 2011). In contrast to KO, the n-3 PUFA in fish oils (FO) are in the TAG form (Winther et al., 2011; Tou et al., 2007).

Several post-prandial and longer-term human studies have investigated the bioavailability (blood levels) of n-3 PUFA from KO and FO in human trials. The results have been inconsistent (Maki et al., 2009; Ramprasath et al., 2013; Schuchardt et al., 2011; Yurko-Mauro et al., 2015). We have investigated the differences in lipidomic profiles between KO and FO supplementations in a post-prandial study (Sung et al., 2019) and a 30-day study (Sung et al., 2020). The post-prandial study showed that eicosapentaenoic acid (EPA) and DHA from KO were preferentially incorporated into plasma phospholipid fraction, whereas following FO consumption EPA and DHA were partitioned towards the TAG fraction (Sung et al., 2019). The 30-day study reported that 77 molecular species of lipids differed significantly between KO and FO. After KO supplementation, the majority of lipid species that increased more than after FO supplementation contained saturated and monounsaturated fatty acids; in contrast, the majority of lipid species that increased more after FO than KO were those containing n-6 PUFA (Sung et al., 2020). The present study is a secondary analysis of the 30-day study, looking specifically at the incorporation of n-3 PUFA into the phospholipid molecular species at days 15 and 30.

The aim was to determine whether the n-3 PUFA from the KO and FO supplements partitioned into plasma phospholipid molecular species to the same extent, following 30 days of supplementation of seven capsules of KO per day (containing a total of 1.27 g n-3 PUFA) or five capsules of FO per day (total of 1.44 g n-3 PUFA).

MATERIALS AND METHODS

Subjects and supplements

This was a randomized crossover study with KO or FO supplementation for 30 days. The supplementation periods were separated by a minimum of 30-day washout (Cicero et al., 2016). During the study period, all participants were instructed to maintain their habitual diet and requested not to consume fish, seafood, or n-3 fortified foods more than once a week. For interventions, participants consumed daily seven 1-g capsules of KO containing 1.27 g of long-chain n-3 PUFA (EPA + DHA + DPAn-3) or five 1-g capsules of FO containing 1.44 g of long-chain n-3 PUFA for 30 days each; these capsule numbers gave the closest possible match for total long-chain n-3 PUFA per group (Sung et al., 2020). Participants were required to attend the clinic at days 0 (baseline), 15, and 30 of each supplementation for blood sample collection. On the evening prior to each clinic visit, participants were required to consume one of the most common low-fat dishes in their diet, avoid drinking alcohol and strenuous physical activities, and fast approximately 10 h overnight. On each study day, standardized procedures were performed where participants arrived at the clinic between 7 and 9 am, and a fasting blood sample (10 ml) was collected via a venepuncture by a qualified practitioner.

A total of 11 healthy women aged between 18 and 50 years with BMI 20–35 (kg/m^2), who had not experienced menopause, were recruited through emails to all Victoria University staff and students, flyer advertisements via the Victoria University Nutritional Therapy Teaching Clinic, community centers and local medical practices. Participants were screened for their suitability for the 30-day study as they completed a medical questionnaire, anthropometric measurements, and the electronic PUFA food frequency questionnaire (FFQ) prior to enrolling into the study. Participants were excluded if their daily long-chain n-3 PUFA was more than 500 mg based on results of the electronic PUFA FFQ (Sullivan et al., 2008). Participants were also excluded if they were cigarette smokers; pregnant or lactating; or had heart, liver, kidney, or inflammatory bowel disease, diabetes; or medications interfering with lipid metabolism or lowering blood lipids; allergy to fish or other seafood; or intake of oily fish more than twice a week or supplements including n-3 fatty acids in the past 4 weeks prior to the study.

The study supplements of KO (Swisse Wellness Pty Ltd., *Euphausia Superba* oil, Victoria, Australia) and FO (Swisse Wellness Pty Ltd., Natural fish oil, Victoria, Australia) were purchased from a local pharmacy and the fatty acid and lipidomic profiles of these oils were analyzed prior to the commencement of the intervention and have been published in full (Sung et al., 2019, 2020).

Ethics approval was obtained from the Victoria University Human Research Ethics Committee (HRE15-031). All experimental procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association. Written informed consent was obtained from all participants prior to the study. This trial was registered with the Australian New Zealand Clinical Trial Registry (ACTRN 12615000472572).

Lipid extraction

Plasma lipids were isolated using a single-phase chloroform: methanol ($CHCl_3$:MeOH) extraction as previously described (Weir et al., 2013). Briefly, plasma samples (10 μl) were extracted in a single-phase extraction with 20 volumes of $CHCl_3$:MeOH (2:1) and 10 μl of an internal standard mix (in $CHCl_3$:MeOH [1:1]) containing between 50 and 1000 pmol each of 23 non-physiological or stable isotope-labeled lipid standards (Weir et al., 2013).

TABLE 1 Lipidomic changes in plasma EPA (20:5) phospholipid molecular species over the 30-day krill oil and fish oil supplementation

	Concentration (pmol/ml)						*p* value							
	Krill oil			Fish oil			Krill oil	Fish oil	Krill oil vs. Fish oil					
EPA (20:5) molecular species	*T* = 0	*T* = 15	*T* = 30	*T* = 0	*T* = 15	*T* = 30	*T*0:T30	*T*0:T30	*T* = 0	*T* = 15	*T* = 30	Time	Treatment	Interaction
PC (38:5)(a)	16,314	16,624	19,209	15,720	18,518	19,096	**0.00**	**0.00**	0.46	0.03	0.89	0.00	0.54	0.09
PC (16:0/20:5)	14,060	48,819	60,310	12,143	48,925	49,923	**0.00**	**0.00**	0.62	0.98	**0.01**	0.00	0.03	0.14
PC (38:5)(b)	9893	24,824	31,028	9354	26,468	26,682	**0.00**	**0.00**	0.78	0.40	**0.03**	0.00	0.22	0.10
PC (O-38:5)	5145	4770	5401	4834	4592	4601	0.33	0.38	0.24	0.50	**0.01**	0.24	0.04	0.22
PC (O-36:5)	4318	3657	4138	4493	4110	4033	0.46	0.07	0.48	0.07	0.67	0.01	0.41	0.28
PC (P-38:5)(a)	2374	2837	3346	2424	2651	2654	**0.00**	0.11	0.72	0.19	**0.00**	0.00	0.02	**0.00**
PE (P-18:0/20:5)	1560	5487	7895	1583	5537	4806	**0.00**	**0.00**	0.97	0.94	**0.00**	0.00	0.00	**0.00**
PE (38:5)(a)	1506	1286	1045	1786	1391	1169	**0.00**	**0.00**	0.03	0.40	0.33	0.00	0.27	0.56
PE (P-16:0/20:5)	864	4171	4574	807	3001	2454	**0.00**	**0.00**	0.68	0.13	**0.00**	0.00	0.00	0.13
PE (O-38:5)(a)	770	881	1138	685	665	539	**0.00**	0.20	0.45	0.06	**0.00**	0.41	0.00	**0.01**
PE (38:5)(b)	725	1457	1495	735	1474	1373	**0.00**	**0.00**	0.94	0.89	0.35	0.00	0.64	0.69
PC (O-40:5)	694	653	741	685	672	662	0.19	0.52	0.79	0.58	**0.03**	0.31	0.33	0.14
PC (P-16:0/20:5)	688	2221	3079	644	2076	2173	**0.00**	**0.00**	0.86	0.55	**0.00**	0.00	0.01	**0.04**
PE (P-18:1/20:5)(a)	578	2242	2646	535	1760	1569	**0.00**	**0.00**	0.56	0.27	**0.01**	0.00	0.01	0.19
LPC (20:5 sn1)	457	2036	2505	439	1823	2111	**0.00**	**0.00**	0.94	0.38	0.12	**0.00**	0.14	0.55
PC (18:2–20:5)	320	1186	1544	347	1078	1124	**0.00**	**0.00**	0.86	0.46	**0.01**	**0.00**	0.10	0.11
PC (P-038:5)(b)	285	504	707	290	499	498	**0.00**	**0.00**	0.92	0.92	**0.00**	**0.00**	**0.05**	**0.01**
PE (16:0–20:5)	165	457	457	165	465	435	**0.00**	**0.00**	1.00	0.89	0.67	**0.00**	0.85	0.92
LPC (20:5 sn2)	148	648	799	147	594	681	**0.00**	**0.00**	1.00	0.44	0.11	**0.00**	0.12	0.50
PE (O-38:5)(b)	141	571	686	131	301	229	**0.00**	**0.02**	0.94	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
PE (P-18:1–20:5)(b)	107	324	425	100	297	308	**0.00**	**0.00**	0.84	0.47	**0.00**	**0.00**	**0.03**	0.09

Notes: Values are expressed as the mean of plasma EPA (20:5) molecular species concentration ($n = 11$). Species shaded in bold significantly different between KO and FO at day 30. Two-way analysis of variance for repeated measurements was performed to analyze supplementation effect over time (interaction time × supplementation), the difference between time points within supplementation, and each time point between the two omega-3 supplementation groups. All *p* values were corrected for multiple comparisons using the Benjamini–Hochberg FDR.

Abbreviations: EPA, eicosapentaenoic acid; LPC, lyso-phosphatidylcholine; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkyl-phosphatidylethanolamine; PE(P), alkenyl-phosphatidylethanolamine; *T*0, baseline; *T*15, 15 days; *T*30, 30 days.

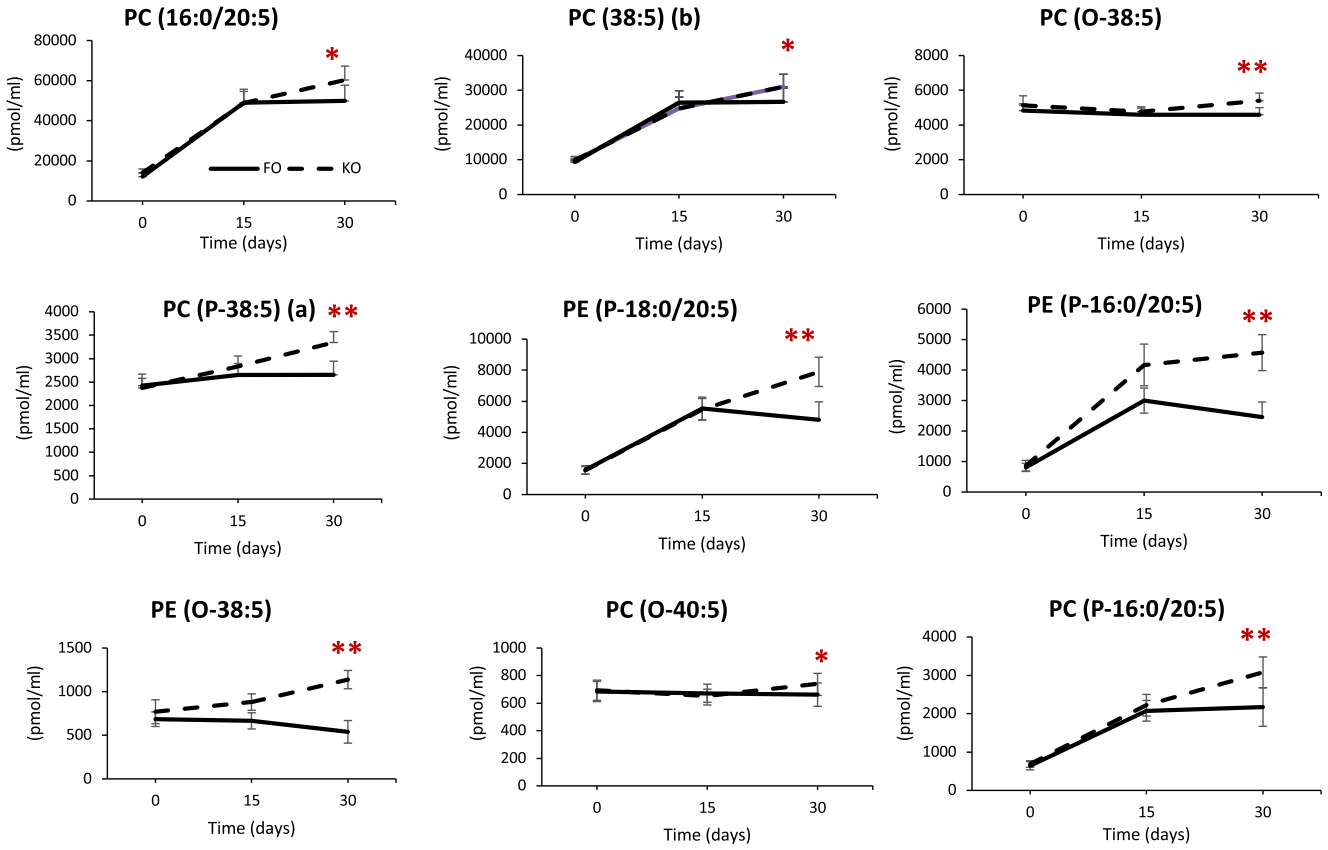

FIGURE 1 Incorporation of EPA (20:5) into nine molecular species with initial concentrations exceeding 500 pmol/ml following the krill oil (KO, dashed lines) or fish oil (FO, solid line) supplementation over 30 days. Values are expressed as mean ± SEM (pmol/ml) ($n = 11$). Two-way analysis of variance for repeated measurements was performed to analyze the difference between each time point between the two omega-3 supplementation groups. The asterisks (*,**) in graphs indicate significant differences ($p \leq 0.05$, $p \leq 0.01$, respectively) between the KO and FO supplementation groups. EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine

Mass spectrometry and lipid analysis

Lipid analysis was performed by high-performance liquid chromatography electrospray ionization-tandem mass spectrometry (HPLC ESI-MS/MS) using an Agilent 1290 HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer. The settings of LC ESI-MS/MS were as follows: gas temperature 150°C, gas flow 17 L/min, nozzle pressure 20 psi, sheath gas temperature 200°C, sheath gas flow 10 L/min, capillary voltage 3500 V and nozzle voltage 1000 V. Liquid chromatography was performed on a Zorbax Eclipse Plus C18, 1.8 μm, 50 × 2.1 mm column (Agilent Technologies) using solvents A and B consisting of water:acetonitrile:isopropanol, 50:30:20 and 1:9:90, respectively, both containing 10 mM ammonium formate. The column was heated to 60°C and the autosampler regulated to 25°C. A total of 522 lipid species were analyzed using dynamic multiple reaction monitoring where data were collected for a retention time window specific to each lipid species. Results from the chromatographic data were analyzed using Mass Hunter Quant where relative lipid abundances were calculated by relating each area under the chromatogram for each lipid species to the corresponding internal standard. Correction factors were applied to adjust for different response factors, where these were known. Species that were chromatographically separated were labeled as such (e.g., PC [16:0–22:6] and PC [18:2–20:4]), whereas species that were mixed isomers were given the standard phospholipid notation (e.g. PC[40:8] was a mixture of 20:4/20:4 and 18:2–22:6) (Huynh et al., 2019). Where structural details were sufficient, lipids were manually annotated as containing long-chain n-3 components (i.e., 20:5 EPA, 22:5 DPA, and 22:6 DHA).

Statistical analysis

Statistical analyses were performed to compare the significant effects of the 30-day supplementation on plasma lipid molecular species between the KO and FO supplementation groups. Values are expressed as mean of concentration ± standard error mean (SEM) for 11 participants. The normality of data distribution was checked using D'Agostino & Pearson normality

TABLE 2 Lipidomic changes in plasma DHA (22:6) phospholipid molecular species over the 30-day krill oil and fish oil supplementation

	Concentration (pmol/ml)						*p* value							
	Krill oil			Fish oil			Krill oil	Fish oil	Krill oil vs. fish oil					
DHA (22:6) molecular species	*T* = 0	*T* = 15	*T* = 30	*T* = 0	*T* = 15	*T* = 30	*T*0:T30	*T*0:T30	*T* = 0	*T* = 15	*T* = 30	Time	Supplement	Interaction
PC (16:0/22:6)	56,421	74,143	84,089	51,481	83,726	86,864	**0.00**	**0.00**	0.24	**0.03**	0.50	**0.00**	0.42	0.06
PC (18:0/22:6)	11,335	16,397	18,919	11,228	17,858	19,596	**0.00**	**0.00**	0.92	0.17	0.52	**0.00**	0.13	0.57
PE (16:0/22:6)	6043	6148	5208	5100	6575	6346	0.15	**0.04**	0.11	0.45	0.05	0.20	0.65	**0.05**
PC (38:6)(a)	5011	8187	9645	5233	7741	7782	**0.00**	**0.00**	0.70	0.44	**0.00**	**0.00**	0.06	0.05
PE (P-16:0/22:6)	3271	5059	5852	3222	4296	3874	**0.00**	0.08	0.89	**0.04**	**0.00**	**0.00**	**0.00**	**0.00**
PC (18:1/22:6)(a)	3245	3698	3874	3184	3589	4083	**0.01**	**0.00**	0.76	0.59	0.31	**0.00**	0.93	0.49
PE (18:0/22:6)	2610	2975	2803	2351	3187	3111	0.43	**0.00**	0.29	0.38	0.21	**0.02**	0.57	0.22
PE (P-18:0/22:6)	2377	3033	3973	2439	3329	3000	**0.00**	0.07	0.83	0.32	**0.00**	**0.00**	0.23	**0.01**
PE (P-18:1/22:6)(a)	2270	3006	3470	2019	2811	2701	**0.00**	**0.00**	0.24	0.36	**0.00**	**0.00**	**0.03**	0.12
PC (O-16:0/22:6)	1832	4631	5523	1855	2852	2631	**0.00**	**0.04**	0.95	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
PC (O-40:7) (b)	1087	1070	1249	1077	1101	1155	**0.01**	0.19	0.86	0.59	0.11	**0.00**	0.37	0.31
LPC (22:6) [sn1]	946	1505	1651	980	1560	1834	**0.00**	**0.00**	0.84	0.74	0.27	**0.00**	0.41	0.78
PC (O-18:0/22:6)	867	1385	1677	884	1122	1132	**0.00**	**0.01**	0.85	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**
PC (P-16:0/22:6)	767	1049	1314	798	1064	1084	**0.00**	**0.00**	0.54	0.77	**0.00**	**0.00**	0.23	**0.00**
PC (O-40:7)(a)	759	1200	1509	734	982	1009	**0.00**	**0.00**	0.74	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**
PC (18:1/22:6)(b)	758	948	1083	696	1070	1153	**0.00**	**0.00**	1.00	0.38	0.38	**0.00**	0.43	0.39
PI (18:0/22:6)	746	1175	1253	764	1241	1220	**0.00**	**0.00**	0.83	0.42	0.69	**0.00**	0.63	0.69
LPE (22:6) [sn1]	695	984	948	719	929	1075	**0.01**	**0.00**	0.79	0.54	0.16	**0.00**	0.54	0.36
PE (40:7)	591	630	558	529	639	603	0.66	0.33	0.41	0.91	0.55	0.19	0.95	0.59
PE (O-16:0/22:6)	522	1464	1711	485	662	559	**0.00**	0.40	0.94	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
PC (16:1/22:6)	511	627	760	454	681	727	**0.00**	**0.00**	0.30	0.33	0.55	**0.00**	0.72	0.33
PE (17:0/22:6)	449	669	766	410	693	752	**0.00**	**0.00**	0.43	0.62	0.77	**0.00**	0.75	0.65
PE (15-MHDA/22:6)	413	519	595	381	596	613	**0.00**	**0.00**	0.53	0.14	0.74	**0.00**	0.58	0.32
PE (P-18:1/22:6)(b)	389	453	578	393	474	476	**0.00**	**0.03**	0.92	0.57	**0.01**	**0.00**	0.38	0.05
LPE (22:6) [sn2]	381	524	523	381	504	536	**0.00**	**0.00**	0.98	0.57	0.72	**0.00**	0.94	0.80
PC (15:0/22:6)	360	469	550	312	534	571	**0.00**	**0.00**	0.75	0.24	0.20	**0.00**	0.85	0.22
PC (14:0/22:6)	355	558	696	337	628	619	**0.00**	**0.00**	0.75	0.24	0.20	**0.00**	0.85	0.22
LPC (22:6) [sn2]	333	518	561	331	530	629	**0.00**	**0.00**	0.97	0.80	0.16	**0.00**	0.47	0.54
PE (O-18:0/22:6)	288	514	626	304	404	332	**0.00**	0.64	0.77	0.07	**0.00**	**0.00**	**0.00**	**0.00**
PE (P-17:0/22:6)	275	371	476	266	344	320	**0.00**	0.13	0.79	0.45	**0.00**	**0.00**	**0.01**	**0.01**
PI (38:6)	274	406	408	285	446	429	**0.00**	**0.00**	0.77	0.28	0.58	**0.00**	0.14	0.84
PC (P-18:1/22:6)	241	301	389	238	301	325	**0.00**	**0.00**	0.87	0.96	**0.00**	**0.00**	0.15	**0.01**

(Continues)

TABLE 2 (Continued)

DHA (22:6) molecular species	Concentration (pmol/ml)						*p* value					
	Krill oil			Fish oil			Krill oil	Fish oil	Krill oil vs. fish oil			
	$T = 0$	$T = 15$	$T = 30$	$T = 0$	$T = 15$	$T = 30$	T0:T30	T0:T30	$T = 0$	$T = 15$	$T = 30$	Time	Supplement	Interaction
PC (P-18:0/22:6)	230	302	399	237	318	328	**0.00**	**0.00**	0.66	0.32	**0.00**	**0.00**	0.44	**0.00**
PE (O-18:1/22:6)	226	443	535	190	287	270	**0.00**	0.10	0.46	**0.00**	**0.00**	**0.00**	**0.00**	**0.01**
PE (P-20:0/22:6)	198	273	311	206	293	277	**0.00**	**0.00**	0.57	0.16	**0.03**	**0.00**	0.87	**0.03**
PC (40:7)	192	168	192	228	177	170	0.96	**0.00**	**0.01**	0.47	0.09	**0.01**	0.54	**0.01**
PE (P-15-MHDA/22:6)	181	240	305	192	244	226	**0.00**	0.15	0.64	0.87	**0.00**	**0.00**	0.16	**0.02**
PE (P-20:1/22:6)	133	159	207	112	155	134	**0.01**	0.44	0.47	0.89	**0.02**	**0.00**	**0.03**	0.21

Notes: Values are expressed as the mean of plasma DHA (22:6) molecular species concentration ($n = 11$). Species shaded in bold significantly different between KO and FO at day 30. Two-way ANOVA for repeated measurements was performed to analyze supplementation effect over time (interaction time × supplementation), the difference between time points within supplementation, and each time point between the two omega-3 supplementation groups. All *p* values were corrected for multiple comparisons using the Benjamini–Hochberg FDR.

Abbreviations: DHA, docosahexaenoic acid; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; PC, phosphatidylcholine; PC(P), alkenyl-phosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkyl-phosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PI, phosphatidylinositol; *T0*, baseline; *T15*, 15 days; *T30*, 30 days.

test. Log-transformation of data was carried out where appropriate. Two-way analysis of variance (ANOVA) for repeated measurements was performed to analyze supplementation effect over time (interaction time × supplementation), differences between time points within supplementation and the same time point between the two omega-3 supplementations. All *p* values were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR). $P < 0.05$ was considered significant. The analyses were performed using GraphPad Prism version 7.01.

RESULTS

A total of 11 healthy women completed the 30-day crossover dietary intervention. The KO intervention was associated with significant increases in plasma EPA concentration at days 5, 10, and 30, compared with FO, resulting in a significantly greater net incremental area under the curve for EPA response following KO consumption compared with FO (Sung et al., 2020). For both treatments, the level of plasma DHA increased significantly over the 30 days, but there was no significant difference between treatments.

EPA-molecular species

A total of 21 EPA-containing phospholipid molecular species were detected in the concentration range of 107–60,310 pmol/ml, as shown in Table 1. The majority of these showed significant increases in concentration at day 30 after both KO supplementation (17/21 increased) and FO supplementation (14/21 increased). At day 30, 14 molecular species in the KO group had significantly higher concentrations compared with the FO group. For the remaining six molecular species from the total of 20, there were no significant differences between the two n-3 oil supplementation groups. Eleven of the 14 which were significantly different were ether-phospholipids, while 3/14 were diacyl phospholipids (PC [16:0–20:5]; PC [38:5] [b]; PC [18:2–20:5]) (Table 1). The nine molecular species whose initial concentrations exceeded 500 pmol/ml and which were significantly different between KO and FO are shown in Figure 1. This illustrates that molecular species following KO supplementation showed progressive increases in concentration over the 30 days compared with the FO group, where in most cases the concentration had plateaued by day 15.

DHA molecular species

A total of 38 DHA-containing phospholipid molecular species were detected in the concentration range of

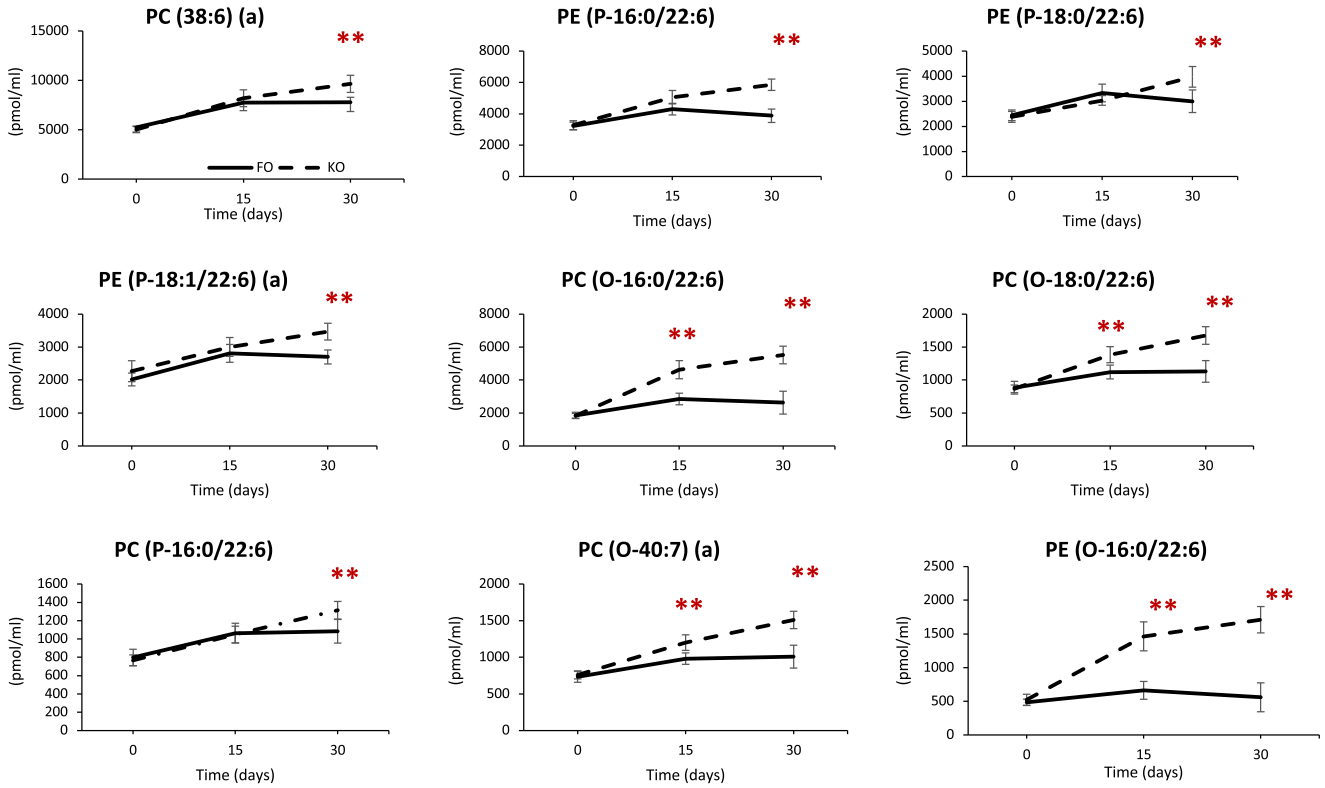

FIGURE 2 Incorporation of DHA (22:6) into nine molecular species with initial concentrations exceeding 500 pmol/ml following the krill oil (KO, dashed lines) or fish oil (FO, solid line) supplementation over 30 days. Values are expressed as mean ± SEM (pmol/ml) ($n = 11$). Two-way analysis of variance for repeated measurements was performed to analyze the difference between each time point between the two omega-3 supplementation groups. The asterisks (*,**) in graphs indicate significant differences ($p \leq 0.05$, $p \leq 0.01$, respectively) between the KO and FO supplementation groups. DHA, docosahexaenoic acid; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine

133–86,864 pmol/ml, as shown in Table 2. The majority of these showed significant increases in concentration at day 30 after both KO supplementation (34/38 increased) and FO supplementation (28/38 increased).

At day 30, 18 molecular species in the KO group had significantly higher concentrations compared with the FO group. For the remaining 20 molecular species from the total of 38, there were no significant differences between the two n-3 oil supplementation groups. Seventeen of the 18 molecular species which had significantly higher concentrations for KO compared with FO groups were ether-phospholipids (Table 2). The nine molecular species whose initial concentration exceeded 500 pmol/ml and which were significantly different between KO and FO are shown in Figure 2. For four molecular species shown in Figure 2, KO was also significantly higher in concentration at day 15 as well as at day 30. This figure shows that molecular species following KO supplementation showed progressive increases in concentration over the 30 days compared with the FO group, where in most cases the concentration had plateaued by day 15.

DPAn-3 molecular species

A total of 12 DPAn-3-containing phospholipid molecular species were detected in the concentration range of 152–6616 pmol/ml (data not shown). The majority of these showed significant increases in concentration at day 30 after both KO supplementation (9/12 increased) and FO supplementation (7/12 increased). At day 30, three molecular species in the KO group had significantly higher concentrations compared with the FO group; these were PE (P-16:0–22:5) (a), PC (P-18:0–22:5), and (PE (O-18:0–22:5) (a).

DISCUSSION

General remarks

The aim of this study was to determine whether the n-3 PUFA from the KO and FO supplements partitioned into plasma phospholipid molecular species in a similar pattern, following 30-day supplementation of KO or FO in healthy young women. Seventy molecular species of

phospholipid containing either EPA, DHA, and DPAn-3 were detected in plasma. A total of 70% of the EPA-containing molecular species increased significantly more after the KO treatment compared with the FO treatment. No EPA species increased more after FO supplementation. A total of 45% of the DHA-containing molecular species increased significantly more after KO treatment than after the FO treatment. No DHA species increased more after FO than after KO supplementation. A total of 25% of the DPA-containing molecular species increased significantly more after KO treatment than after the FO treatment. Finally, the majority (89%) of the molecular species that increased more after KO than FO were choline or ethanolamine ether phospholipids (alkyl- and alkenyl-phospholipids).

These results clearly showed that after KO supplementation EPA, DHA, and DPAn-3 were enriched in specific plasma lipid species compared with FO. This is a novel finding. We note that our previous 5-h postprandial study in young women reported that two choline ether phospholipid molecular species (PC[O-34:4] and PC[0–36:5]) were significantly increased after KO compared with FO (Sung et al., 2019).

Ether-containing phospholipids are minor components in plasma and consist of either alkyl or alkenyl phosphatidylcholine and phosphatidylethanolamine (the latter being referred to as plasmalogens). These have only been studied recently because techniques such as lipidomics have allowed detection and quantitation of these and other minor lipid classes (Meikle et al., 2015).

Wiesner et al. (2009) reported that ethanolamine plasmalogens (PlsEtn) accounted for 1.1% of total plasma polar lipids in fasting blood, with 12% of these being in VLDL, 30% in LDL and 60% in HDL. Another study reported that arachidonic acid was the major PUFA associated with both phosphatidylcholine plasmalogen (PlsCho) and PlsEtn in human plasma (Otoki et al., 2017).

PtdCho is the major lipid species in human plasma and this study found that both KO and FO significantly increased molecular species of PtdCho containing EPA and DHA, showing that there was significant turnover of the 2-position PUFA in plasma PtdCho species. For example, the three main EPA-containing PtdCho species were PC(38:5)(a), PC(16:0/20:5) and PC(38:5)(b), and the two main DHA-containing PtdCho species were PC(16:0/22:6) and PC(18:0/22:6). The increases in these five species from baseline to day 30 ranged from 1.17- to 4.28-times; however, for only two of these PtdCho species (PC (16:0/20:5) and PC (38:5) (b)) were there differences between KO and FO treatment, both in favor of the KO increases being significantly greater than the FO. A possible reason why PtdCho (16:0/20:5) species showed differences between KO and FO is that this species was the major PtdCho species in KO (15% total phospholipid species) (Sung et al., 2019); however, it is not certain that dietary PtdCho molecular species retain their structure following digestion and absorption, and transport from liver (Sung et al., 2020).

Both KO and FO supplementations significantly increased the concentration of molecular species of ether phospholipids containing EPA and DHA after 30 days. These PUFA are in the 2-position of the ether phospholipids and these data show that there was significant turnover of 2-position fatty acids even by day 15 in ether phospholipids. The most interesting finding from this study was that ether phospholipids in plasma showed significantly greater incorporation of EPA and DHA following KO than FO treatment. We propose that ether phospholipids present in the KO contributed to increased ether phospholipid concentrations in plasma, since circulating plasmalogens are either derived from dietary sources and/or are synthesized in the liver and gastrointestinal epithelium (Roels et al., 1987, Astarita et al., 2010, Paul et al., 2021). In the present study, alkyl-phosphatidylcholine (PakCho) and lyso-PakCho were the main species detected in the KO supplement, accounting for 7% of the choline phospholipids, with PC(O-36:5), PC(O-16:0–22:6), LPC(O-16:1), LPC(O-18:1), and PC(O-32:1) accounting for 6% (Sung et al., 2019).

What is the evidence that dietary plasmalogens are bioavailable?

There have been few studies which have examined the digestion, absorption, and transport of ether-phospholipids, including plasmalogens, thus, many questions about these processes are still unresolved. Oral gavage of mice with PlsEtn isolated from oysters or ascidians was accompanied by increases in the plasma levels, over a 4-h period, of PlsEtn species such as PlsEtn(18:0/22:6) and PlsEtn(18:0/20:5), which were the main species in the purified PlsEtn fed (Yamashita et al., 2021). They also reported a significantly increased plasma level of PlsEtn species if the 1-lyso-PlsEtn and FFA derived from the PlsEtn were gavaged together, and they inferred from their data that PlsEtn was digested to 1-lyso-PlsEtn prior to absorption. In a separate study, Takahashi et al. (2020) used lymph canulation in rats and everted sac studies to examine the absorption of PlsEtn species rich in PlsEtn (18:0/22:6) (94%), but also containing PlsEtn (18:0/20:4) and PlsEtn(18:0/18:1). They reported that the main species appearing in the lymph were PlsEtn (18:0/22:6) and PlsEtn(18:0/20:4), in equal quantity, with little evidence of any PlsEtn(18:0/18:1). Furthermore, they detected the appearance of PlsCho (18:0/20:4), a species which had not been administered. These data suggested that there were structural changes in PlsEtn during digestion and absorption including re-esterification of the 2-position to 20:4 species and conversion of ethanolamine to choline base of

the phospholipids. Another rat study reported that when 0.1% of the diet (weight %) was fed as plasmalogens from chicken skin (PlsEtn), the relative compositions of erythrocyte PlsEtn and PtdEtn increased after 4 weeks (Mawatari et al., 2012). Paul et al. (2021) recently showed that plasma accumulated highly polyunsaturated species of alkyl and alkenyl PtdCho and PtdEtn following the consumption of alkylglycerols containing fatty acids with fewer double bonds, indicating that rearrangement of the fatty acyl component of alkyl lipids occurs following digestion and absorption. Furthermore, it was clear that alkyl species had been metabolized to plasmalogens (PlsEtn and PlsCho).

Novelty of this study

We previously reported on changes in the plasma lipidome from the same study (Sung et al., 2020). In that study, the focus was on the entire lipidome including TAG, polar lipids, and minor lipid species including acylcarnitines, gangliosides, sulfatides, and cholesterylesters. The conclusion was that KO treatment significantly increased n-3-containing (such as PC [16:0–20:5]), saturated and monounsaturated-containing molecular species (such as lysophosphatidylinositol [18:1]) compared with FO, while the main effects of FO were on n-6-containing species (such as DG[16:0–20:4]), saturated and monounsaturated-containing species (such as PS[36:1]). The details of changes in concentration of lipid classes and molecular species are available in supplementary files in Sung et al. (2020). In the present secondary analysis, the emphasis was entirely on phospholipid molecular species enriched in n-3 PUFA. The novelty of this study is that we showed that the majority (89%) of the differentiated molecular species were choline and ethanolamine ether-phospholipids, with KO treatment resulting in an enrichment of n-3 PUFA in molecular species of ether-phospholipids compared with the FO treatment.

Why does the concentration of ether phospholipids continue to increase after KO, but plateaus after FO?

The majority of the ether phospholipids species after KO supplementation showed increased concentrations with time (Tables 1 and 2), in contrast to the FO supplement where the concentrations plateaued between days 15 and 30. This suggests that the continued input of ether phospholipids in the KO had not yet led to a steady state after 30 days.

Significance of ether phospholipids

Plasmalogens are important structural phospholipids in the cell membranes, especially brain, erythrocytes, skeletal muscle and spermatozoa, and they are also precursors of secondary messages, via the specialized lipid mediators formed from arachidonic acid and DHA which are found in the 2-position of the ether phospholipids (Su et al., 2019). Plasmalogens also have reported functions as antioxidants, mediators of membrane dynamics and are involved in membrane fusion, ion transport, cholesterol efflux, membrane-bound enzyme activity, and diffusion of signal-transduction molecules (Su et al., 2019, Pike et al., 2002, Honsho et al., 2008, Hossain et al., 2020). Limited studies have shown positive therapeutic outcomes with plasmalogen interventions in subjects with Alzheimer's Disease (AD) and in rodent models of AD (Su et al., 2019).

In conclusion, these data reveal that molecular species analysis of plasma phospholipids following 30 days of consumption of a marine oil rich in phospholipids, including ether phospholipids (such as KO) showed enrichment of n-3 PUFA in ether-phospholipid species compared with a TAG-rich marine oil (FO). Based on the results reported here, future studies should examine in detail the relative impact of KO treatment on n-3 PUFA in TAG, DAG as well as polar lipid molecular species in male and female subjects of different ages. Furthermore, especially needed are studies using purified preparations of plasmalogens from marine sources to understand further details on the digestion, absorption, subsequent metabolism, and physiological significance of dietary plasmalogens in human subjects.

ACKNOWLEDGMENTS

Ms Shaan Naughton, College of Health and Biomedicine, Victoria University, and A/Prof. Patrick McLaughlin for their expert advice on statistical analysis. The lipidomic analyses were conducted by Professor Peter Meikle and his team at the Baker Heart and Diabetes Institute (Melbourne). Their support including that of Natalie Mellett is gratefully acknowledged.

CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Andrew J. Sinclair and Xiao Q Su conceived and designed the study, Andrew J. Sinclair wrote the first draft of the manuscript, Hyunsin (Hedy) Sung carried out the research and analyzed the data. All authors contributed to and approved the final draft of the manuscript.

ORCID

Andrew J. Sinclair https://orcid.org/0000-0002-9515-9721

Xiao Q Su https://orcid.org/0000-0001-8178-406X

REFERENCES

Astarita G, Jung KM, Berchtold NC, Nguyen VQ, Gillen DL, Head E, et al. Deficient liver biosynthesis of docosahexaenoic acid

correlates with cognitive impairment in Alzheimer's disease. PLoS One. 2010;5(9):e12538. https://doi.org/10.1371/journal.pone.0012538

Cicero AF, Rosticci M, Morbini M, Cagnati M, Grandi E, Parini A, et al. Lipid-lowering and anti-inflammatory effects of omega 3 ethyl esters and krill oil: a randomized, cross-over, clinical trial. Arch Med Sci. 2016;12(3):507–12. https://doi.org/10.5114/aoms.2016.59923

Honsho M, Yagita Y, Kinoshita N, Fujiki Y. Isolation and characterization of mutant animal cell line defective in alkyl-dihydroxyacetonephosphate synthase: localization and transport of plasmalogens to post-Golgi compartments. Biochim Biophys Acta. 2008;1783(10):1857–65. https://doi.org/10.1016/j.bbamcr.2008.05.018

Hossain MS, Mawatari S, Fujino T. Biological functions of Plasmalogens. Adv Exp Med Biol. 2020;1299:171–93. https://doi.org/10.1007/978-3-030-60204-8_13

Huynh K, Barlow CK, Jayawardana KS, Weir JM, Mellett NA, Cinel M, et al. High-throughput plasma lipidomics: detailed mapping of the associations with cardiometabolic risk factors. Cell Chemical Biology. 2019;26:71–84.e74.

Maki KC, Reeves MS, Farmer M, Griinari M, Berge K, Vik H, et al. Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. Nutr Res. 2009;29(9):609–15. https://doi.org/10.1016/j.nutres.2009.09.004

Mawatari S, Katafuchi T, Miake K, Fujino T. Dietary plasmalogen increases erythrocyte membrane plasmalogen in rats. Lipids Health Dis. 2012;11:161. https://doi.org/10.1186/1476-511X-11-161

Meikle PJ, Barlow CK, Mellett NA, Mundra PA, Bonham MP, Larsen A, et al. Postprandial plasma phospholipids in men are influenced by the source of dietary fat. J Nutr. 2015;145(9):2012–8. https://doi.org/10.3945/jn.115.210104

Otoki Y, Kato S, Kimura F, Furukawa K, Yamashita S, Arai H, et al. Accurate quantitation of choline and ethanolamine plasmalogen molecular species in human plasma by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2017;134:77–85. https://doi.org/10.1016/j.jpba.2016.11.019

Paul S, Smith AAT, Culham K, Gunawan KA, Weir JM, Cinel MA, et al. Shark liver oil supplementation enriches endogenous plasmalogens and reduces markers of dyslipidemia and inflammation. J Lipid Res. 2021;62:100092. https://doi.org/10.1016/j.jlr.2021.100092

Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. Biochemistry. 2002;41(6):2075–88. https://doi.org/10.1021/bi0156557

Ramprasath VR, Eyal I, Zchut S, Jones PJ. Enhanced increase of omega-3 index in healthy individuals with response to 4-week n-3 fatty acid supplementation from krill oil versus fish oil. Lipids Health Dis. 2013;12:178. https://doi.org/10.1186/1476-511X-12-178

Roels F, Verdonck V, Pauwels M, De Catte L, Lissens W, Liebaers I, et al. Light microscopic visualization of peroxisomes and plasmalogens in first trimester chorionic villi. Prenat Diagn. 1987;7(7):525–30. https://doi.org/10.1002/pd.1970070709

Schuchardt JP, Schneider I, Meyer H, Neubronner J, von Schacky C, Hahn A. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations—a comparative bioavailability study of fish oil vs. krill oil. Lipids Health Dis. 2011;10:145. https://doi.org/10.1186/1476-511X-10-145

Su XQ, Wang J, Sinclair AJ. Plasmalogens and Alzheimer's disease: a review. Lipids Health Dis. 2019;18(1):100. https://doi.org/10.1186/s12944-019-1044-1

Sullivan BL, Brown J, Williams PG, Meyer BJ. Dietary validation of a new Australian food-frequency questionnaire that estimates long-chain n-3 polyunsaturated fatty acids. Br J Nutr. 2008;99(3):660–6. https://doi.org/10.1017/S0007114507837408

Sung HH, Sinclair AJ, Huynh K, Smith AAT, Mellett NA, Meikle PJ, et al. Krill oil has different effects on the plasma Lipidome compared with fish oil following 30 days of supplementation in healthy women: a randomized controlled and crossover study. Nutrients. 2020;12(9):2804. https://doi.org/10.3390/nu12092804

Sung HH, Sinclair AJ, Huynh K, Smith AT, Mellett NA, Meikle PJ, et al. Differential plasma postprandial lipidomic responses to krill oil and fish oil supplementations in women: a randomized cross-over study. Nutrition. 2019;65:191–201. https://doi.org/10.1016/j.nut.2019.03.021

Takahashi T, Kamiyoshihara R, Otoki Y, Ito J, Kato S, Suzuki T. Structural changes of ethanolamine plasmalogen during intestinal absorption. Food Funct. 2020;11(9):8068–76. https://doi.org/10.1039/d0fo01666g

Tou JC, Jaczynski J, Chen YC. Krill for human consumption: nutritional value and potential health benefits. Nutr Rev. 2007;65(2):63–77. https://doi.org/10.1111/j.1753-4887.2007

Weir JM, Wong G, Barlow CK, Greeve MA, Kowalczyk A, Almasy L, et al. Plasma lipid profiling in a large population-based cohort. J Lipid Res. 2013;54(10):2898–908. https://doi.org/10.1194/jlr

Wiesner P, Leidl K, Boettcher A, Schmitz G, Liebisch G. Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry. J Lipid Res. 2009;50(3):574–85. https://doi.org/10.1194/jlr.D800028-JLR200

Winther B, Hoem N, Berge K, Reubsaet L. Elucidation of phosphatidylcholine composition in krill oil extracted from Euphausia superba. Lipids. 2011;46(1):25–36. https://doi.org/10.1007/s11745-010-3472-6

Yamashita S, Fujiwara K, Tominaga Y, Nguma E, Takahashi T, Otoki Y, et al. Absorption kinetics of ethanolamine Plasmalogen and its hydrolysate in mice. J Oleo Sci. 2021;70(2):263–73. https://doi.org/10.5650/jos.ess20223

Yurko-Mauro K, Kralovec J, Bailey-Hall E, Smeberg V, Stark JG, Salem N Jr. Similar eicosapentaenoic acid and docosahexaenoic acid plasma levels achieved with fish oil or krill oil in a randomized double-blind four-week bioavailability study. Lipids Health Dis. 2015;14:99. https://doi.org/10.1186/s12944-015-0109-z

How to cite this article: Sung H(H), Sinclair AJ, Su XQ. Enrichment of n-3 containing ether phospholipids in plasma after 30 days of krill oil compared with fish oil supplementation. Lipids. 2022;57:115–24. https://doi.org/10.1002/lipd.12335