

<https://doi.org/10.1038/s41538-026-00727-5>

Effects of Antarctic krill oil on lipid profiles and SPM levels in rats over time

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Antarctic krill oil (KO) is a rich source of omega-3 polyunsaturated fatty acids (PUFAs). Endogenous PUFA-derived specialized pro-resolving mediators (SPMs) have garnered attention due to their beneficial effects on body, especially the cardiovascular system. This study integrated non-targeted and targeted lipidomics to investigate KO's time-dependent effects on the comprehensive lipid profile and SPMs in rats. After 1- and 6-week supplementation, KO significantly altered lipid profiles, reducing arachidonic acid (ARA, 20:4)-containing lipids while elevating eicosapentaenoic acid (EPA, 20:5)/docosahexaenoic acid (DHA, 22:6)-containing lipids. Targeted analysis identified and quantified 33 PUFA-derived oxylipins, including derivatives of ARA, 8 derivatives of EPA, and 13 derivatives of DHA. Notably, KO consumption substantially decreased pro-inflammatory oxylipins like LTB₄, PGE₂, and TXB₂, while increasing anti-inflammatory LXA₄ and SPMs such as RvE1, RvE2, RvD1, RvD4, and MaR1. Long-term intake amplified SPM accumulation, suggesting temporal regulation. These findings elucidate KO's potential mechanism in inflammation management through lipidome remodeling, supporting its application in functional foods for metabolic health enhancement.

Antarctic krill oil has been reported to exert antioxidant, anti-inflammatory, and heart-protective properties¹. It is a rich source of long-chain omega-3 (ω -3) polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6)². Notably, krill oil is phospholipid-rich, with phospholipids accounting for >55% of total lipids, predominantly phosphatidylcholine (PC)³. Unlike most other oils of marine origin (such as fish oil), which primarily contains ω -3 fatty acids as triacylglycerols or fatty acid ethyl esters, krill oil provides a major part of these fatty acids in the form of phospholipids. Long chain ω -3 PUFAs embedded in phospholipids may facilitate the passage of fatty acids through the intestinal wall. Comparative studies have reported EPA and DHA from krill oil may exhibit higher bioavailability than those from fish oil⁴; however, results may depend on methodological factors, including study design, dose matching and the bioavailability marker assessed (e.g., plasma phospholipids versus erythrocyte ω -3 index)^{5,6}. Therefore, while krill oil is frequently considered a source of ω -3 PUFAs,

further better-designed clinical studies are needed to define effective dosing and substantiate clinical efficacy.

Although ω -3 PUFA supplementation has been widely investigated for anti-inflammatory and cardiometabolic benefits⁷, large trials and evidence syntheses report variable effects on systemic biomarkers and major outcomes^{8,9}. It has been found that some of the physiological effects of PUFAs is mediated by their oxidative metabolites^{10,11}. These oxidative metabolites, known as oxylipins, are produced through mono- or dioxygenase-dependent reactions, which act as lipid mediators involved in various physiological processes¹². The typical pathway for oxylipin biosynthesis begins with the release of PUFAs from membrane phospholipids through the action of phospholipase A₂ (PLA₂) enzymes. After release, non-esterified PUFAs undergo oxidation through the action of three main enzyme systems: lipoxygenases (LOXs)—primarily 5-LOX, 12-LOX, and 15-LOX—encoded by *ALOX* genes; cyclooxygenases (COX-1 and COX-2); and cytochrome P450 (CYP450) enzymes¹³. Notably, the long-chain ω -6

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PUFA arachidonic acid (ARA, 20:4) serves as a major precursor for the generation of oxylipins, such as prostaglandins (PGs), leukotrienes (LTs), and thromboxane (TXs)¹⁴. In addition to these eicosanoids, specialized pro-resolving mediators (SPMs) represent another group of lipid mediators recognized for their potent biological activities in regulating inflammation¹⁵. These compounds possess the capability to oppose the formation and activity of pro-inflammatory mediators, restrict further neutrophil infiltration at sites of inflammation, promote phagocytosis to eliminate apoptotic cells, cellular debris, and bacteria, and regulate the activation of immune cells¹⁶. Additionally, the SPMs are highly effective in promoting wound repair and tissue regeneration, as well as reducing inflammatory pain¹⁷. SPMs are classified into four distinct families, namely lipoxins (LXs), resolvins (both E- and D-series Rvs), protectins (PDs), and maresins (MaRs). Studies indicated that the level of SPMs and/or related metabolic pathways are dysregulated in a variety of diseases, such as chronic inflammatory conditions (e.g., rheumatoid arthritis and periodontitis), neurodegenerative disease, cardiovascular diseases (e.g., atherosclerosis, myocarditis and heart failure), and metabolic disorders (e.g., diabetes and obesity)^{15,18}. Therefore, modulating the body's SPM levels is also viewed as a promising method for resolving inflammation¹⁹.

Studies have demonstrated that consuming marine oils boosts the levels of EPA, DHA and EPA/DHA-derived oxylipins^{13,20,21}. Nevertheless, the majority of current studies examining the impact of ω -3 PUFAs intake on oxylipin levels in healthy organisms primarily reported alterations in fatty acids and more abundant oxylipins such as HEPES, HDHAs, etc. Data on SPMs remain limited, largely because their low physiological concentrations challenge detection and quantification. For instance, Naoe et al. investigated the lipid profiles in the plasma and tissues of mice following dietary intake of ethyl ester forms of ARA, EPA, or DHA. The oxylipins they identified and quantified were predominantly PGs, TXs, and fatty acid hydroxides, with only 2 SPMs detected²². Currently, there is a scarcity of research addressing the effects of krill oil supplementation on SPM levels in healthy organisms.

The production of SPMs involves multiple enzymatic processes and cellular interactions, resulting in lower levels of SPMs compared to their mono-hydroxylated precursors²³. Consequently, the reliable detection of SPMs requires not only highly sensitive and selective analytical platforms, but also adherence to international guidelines and the technical recommendations for oxylipin analysis, including appropriate limits of detection and quantification (LOD/LOQ), chromatographic resolution of isomeric species, and confirmatory MS/MS identification criteria²⁴. Currently, the identification and quantification of SPMs and other oxylipins are primarily achieved using liquid chromatography (LC) combined with electrospray ionization and tandem mass spectrometry (MS/MS)^{25,26}. Nevertheless, despite the availability of advanced LC-MS/MS platforms, robust detection and quantification of many SPMs remain challenging, primarily because these mediators are typically present at very low endogenous concentrations and are prone to analytical confounders (e.g., matrix effects and isomeric interferences). Consequently, only a limited number of studies have reported absolute SPM concentrations in vivo, and the validity of some reported findings has been questioned. In particular, Dr Schebb et al. (and related methodological critiques) emphasized that insufficient compliance with standardized analytical criteria can lead to false-positive SPM assignments^{27,28}. In this study, we employed a Qtrap 6500 mass spectrometer to analyze SPMs and other oxylipins, while carefully considering these methodological challenges and adhering to current international guidelines for oxylipin detection and identification.

In present investigation, we thoroughly analyzed the effects of short-term (1 week) and long-term (6 weeks) krill oil dietary supplementation on lipid metabolism levels in the peripheral blood of rats using non-targeted lipidomics. Additionally, we employed targeted lipidomics to evaluate the impact of exogenous krill oil supplementation on the concentrations of SPMs and related oxylipins in rats. We also aimed to unravel the temporal dynamics between krill oil intervention and SPM levels within the organism. This study underscored the potential of Antarctic krill oil as a dietary

intervention to support homeostasis and improve overall health, offering valuable insights for its integration into daily diets.

Results

Changes in lipid profiles after dietary supplementation with krill oil

Non-targeted lipid metabolomics was employed to analyze the changes in lipid metabolite levels in rats following both long-term and short-term dietary supplementation with krill oil. The distribution (average retention time and *m/z* value) of features extracted from negative and positive ion modes were presented in Fig. 1A, B, respectively. In total, more than 16,000 ions were extracted, with 3592 ions from negative ion mode and 12,469 ions from positive ion mode. Figure 1C demonstrated that 695 unique lipid species from six lipid classes were successfully annotated. These included 38.3% glycerolipids (GLs), 26.9% glycerophospholipids (GPs), 15.8% sphingolipids (SPs), 10.2% fatty acyls (FAs), 8.5% sterol lipids (STs) and 0.3% prenol lipids (PRs). As depicted in Fig. 1D, in positive ion mode, 427 lipid species covering six classes and 17 subclasses were identified, encompassing diglycerides (DGs), lysophosphatidylcholines (LPCs), monogalactosyldiacylglycerols (MGDGs), sphingomyelins (SMs), PCs, cholesteryl esters (CEs), and (CARs), among others. Among these, triacylglycerols (TGs) were the most abundant subclass, with 126 molecular species, followed by LPCs at 67 and MGDGs at 54. In negative ion mode, a total of 268 lipids were detected, belonging to five classes and 20 subclasses, including PCs, phosphatidylethanolamine (PEs), lysophosphatidylethanolaminesulfatides (LPEs), sulfoglycosphingolipids (SHex-Cers), seminominal lipids (SMDGDs), free fatty acids (FFAs), fatty acid ester of hydroxyl fatty acid (FAHFA), acylhexosyl sitosterol (AHexSIS), and others. Of these, PCs were the most abundant subclass, with 48 molecular species, followed by SHexCer and SMGDG, which identified 45 species and 44 species, respectively.

The lipidomic changes in rats after dietary supplementation with krill oil were analyzed using an unsupervised PCA model. In Fig. 1E, five principal components accounted for 94.3% of the cumulative variance in the lipid dataset. As demonstrated in the score plot (Fig. 1F), the three groups exhibited complete separation even in the unsupervised mode. The results indicated that dietary supplementation with krill oil significantly altered the lipidomics in rats. Of the 695 lipid molecules detected, 424 exhibited significant differences ($p < 0.05$), accounting for 61.01% of the total. Among these ions, the distribution of the ten subclasses with the highest abundance across the three groups was illustrated in the sankey diagram (Fig. 2A). PC, LPC, and TG were the primary differential subclasses. The heatmap (Fig. 2B) displayed the top 50 significantly different lipid molecules (ordered from smallest to largest p value).

To better understand the changes in rat lipid composition after dietary supplementation with krill oil, volcano plots (Fig. 2C–E) were plotted for the three comparative groups based on the criteria of a \log_2 (fold change, (FC)) ≥ 1 or ≤ -1 , and a p value < 0.05 . In the comparisons of KO1 versus ND, KO6 versus ND, and KO6 versus KO1, a total of 240, 312, and 271 lipid metabolites, respectively, were identified as different ($p < 0.05$). Compared to the ND group, the KO1 group exhibited significant alterations with 50 lipid molecules increasing and 10 decreasing. Following 6 weeks of supplementation, 47 and 56 lipid metabolites were significantly up-regulated and down-regulated, respectively. The comparison between KO6 and KO1 groups revealed distinct differences in the effects of long-term and short-term interventions on serum lipid metabolism. Specifically, in the KO6 group, 12 lipid metabolites were significantly up-regulated, while 44 were significantly down-regulated relative to the KO1 group. Additionally, PLS-DA models were created for the three comparison groups, as depicted in Fig. S1. Differential lipid metabolites were further filtered based on the VIP > 1 criterion, with the significantly upregulated and downregulated metabolites between the comparison groups detailed in Table S1. Compared to the ND group, the lipid molecules exhibiting the highest fold changes in upregulation following krill oil intervention were as follows (with the data enclosed in parentheses indicating the FC observed after 1 week and 6 weeks of

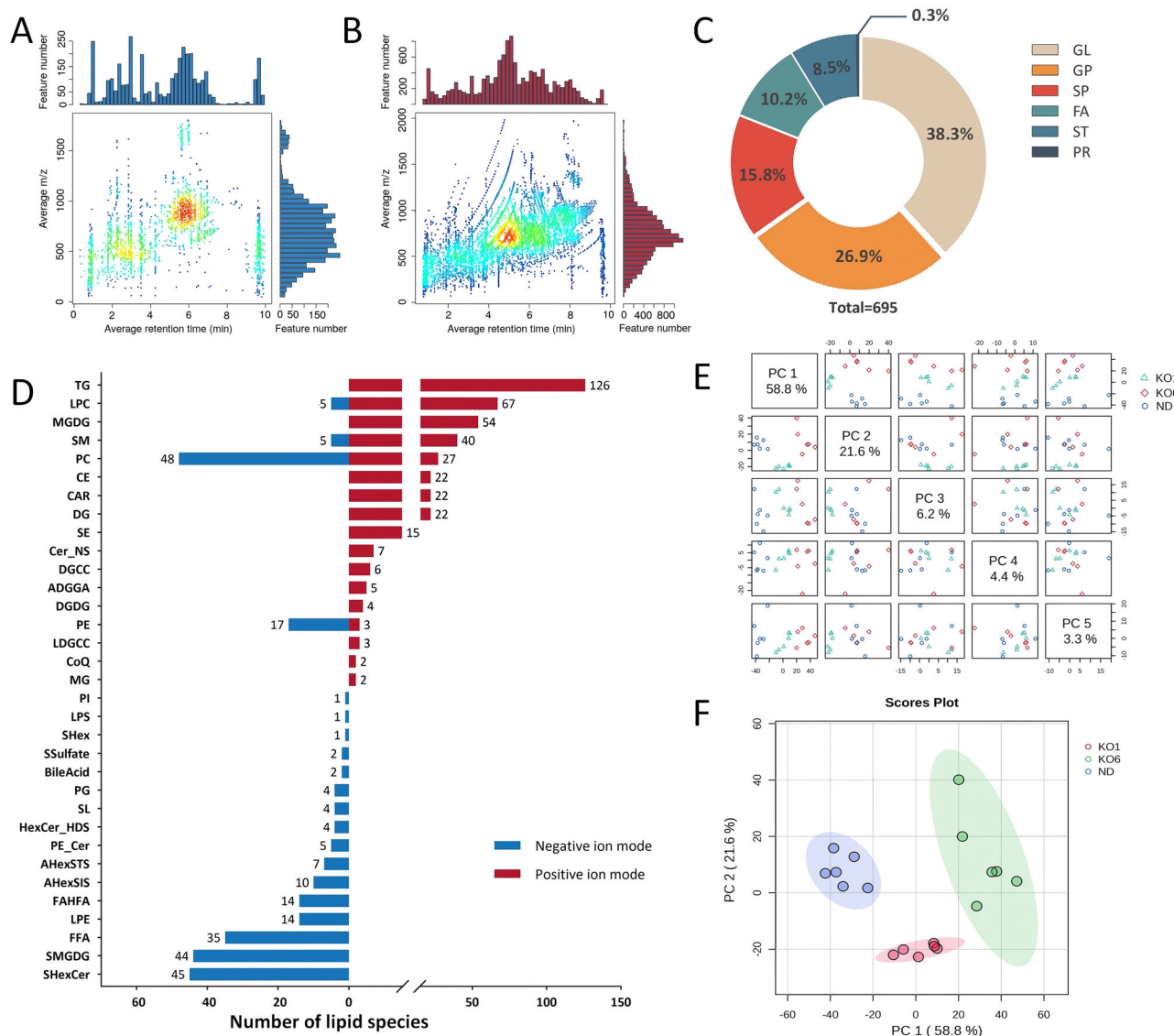


Fig. 1 | Global characterization of the lipidomics dataset and unsupervised PCA overview across the three groups. Distribution (average retention time and *m/z* value) of detected features in the negative (A) and positive (B) ionization modes. C The proportion of lipid classes among all annotated lipids (total = 695). D Numbers of identified lipid species across lipid subclasses in negative (blue) and

positive (red) ion modes. E PCA pair plot based on the intensities of lipid species. F PCA score plot showing the overall clustering/separation trend among ND, KO1, and KO6 samples. The PC1 (58.8%) explains the majority of variance, primarily driven by changes in TG, PC, and LPC species. GL glycerolipid, GP glycerophospholipid, SP sphingolipid, FA fatty acyl, ST sterol lipid, PR prenol lipid.

intervention, respectively): FAHFA 20:5_20:4 (24.76, 62.10), FA 20:5 (7.15, 24.83), SHexCer 36:0;3O (13.20, 12.74), PC 16:0_20:5 (13.19, 10.53), MGDG O-28:4_20:5 (6.65, 11.99), CE 20:5 (9.69, 11.22), and ADGGA 18:2_14:0_14:1 (8.12, 10.96). Conversely, the lipid molecules showing the highest fold changes in downregulation were as follows: SHexCer 27:3;3O (0.19, 0.83), TG 16:0_16:1_18:2 (0.71, 0.25), TG 18:0_20:4_24:1 (0.29, 0.28), MGDG O-28:2_16:0 (0.86, 0.30), TG 15:0_18:1_18:2 (0.85, 0.3), TG 16:0_18:2_18:3 (0.53, 0.3), TG 18:1_18:1_18:2 (0.91, 0.30), and TG 16:0_20:1_26:1 (1.33, 0.3).

The Venn diagram (Fig. 2F) illustrated the overlap of significantly altered lipid molecules across the three comparison groups. We found that 40 lipid molecules, such as CE20:5, DG18:2_20:5, DG18:2_22:6, DG20:4_20:5, LPC20:5, LPE20:5, MGDGO-22:6_28:5, MGDGO-28:5_20:5, PC16:0_20:5, TG18:2_20:5_22:6, TG20:4_20:4_22:6, CAR20:4, PC16:0_20:4;O, TG18:0_20:4_24:1, FA20:5, FAHFA20:5_20:4, and SMGDGO-8:0_17:2, etc. were significantly regulated during both 1-week and 6-week krill oil supplementation. Among them, three lipid molecules—FA20:5, FAHFA20:5_20:4, and SMGDGO-8:0_17:2—were present in all

three comparative groups. The violin plots for these three compounds were shown in Fig. S2. The KEGG enrichment analysis across the three comparison groups (Fig. S3) indicated that several pathways associated with lipid metabolism were enriched, notably the glycerophospholipid metabolism, glycerolipid metabolism, sphingolipid metabolism, arachidonic acid metabolism, linoleic acid metabolism, and alpha-linoleic acid metabolism pathway. Based on the analysis presented, it was clear that dietary intervention with Antarctic krill oil significantly altered the lipid profile in rats, particularly lipid molecules containing PUFAs. Moreover, these alterations were markedly influenced by the duration of the intervention.

Analysis of lipid molecules contained ARA, EPA and DHA

To gain a clearer understanding of SPM precursor levels within the body, we conducted a further analysis of lipid molecules that contain the three primary types of PUFAs: ARA, EPA, and DHA. Among the lipid molecules detected, 42 contained 20:4 (predominantly ARA, 20:4n-6), 28 contained 20:5 (predominantly EPA, 20:5n-3), and 26 contained 22:6 (predominantly DHA, 22:6n-3). The heatmaps (Fig. 3A–C) displayed all lipid molecules that

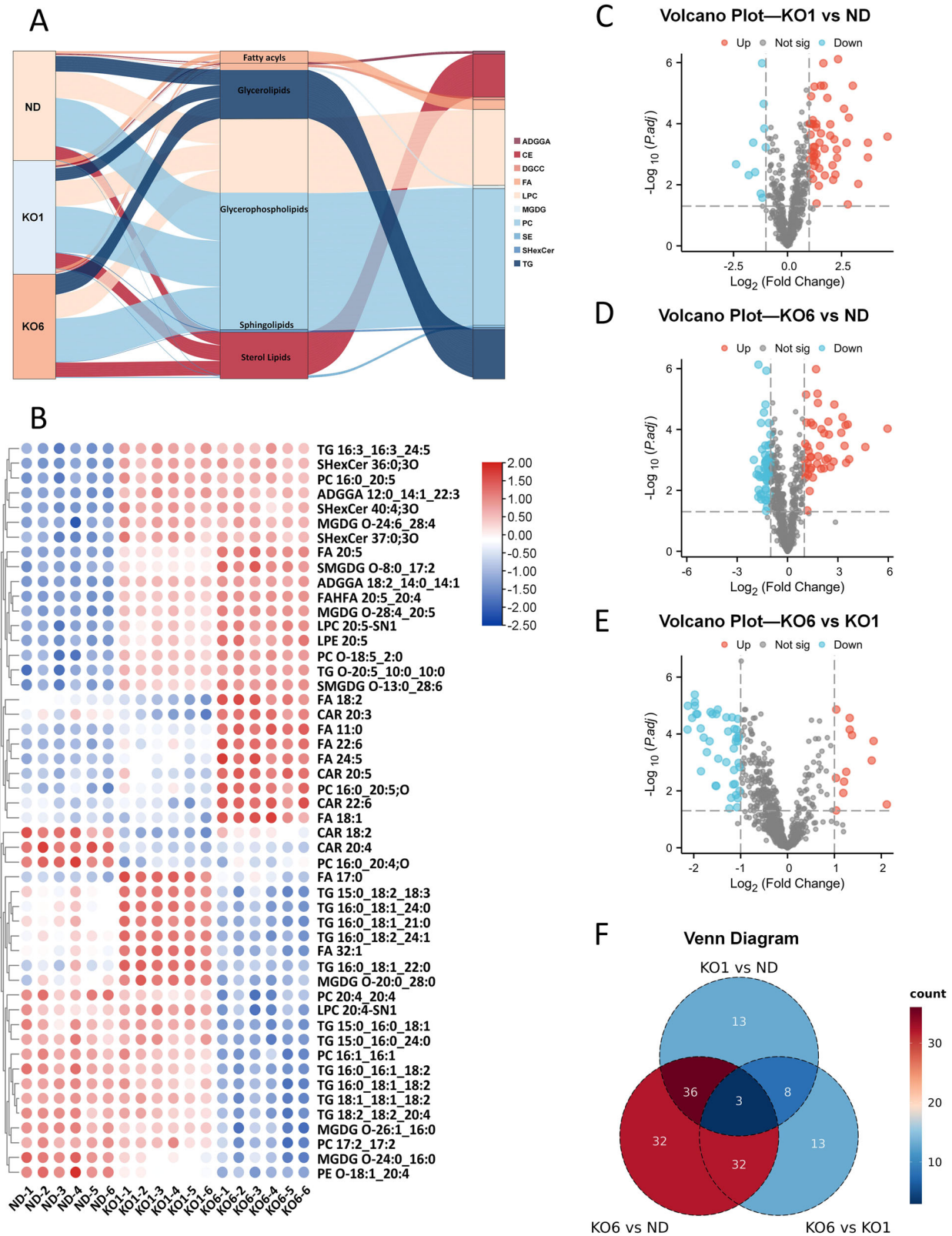


Fig. 2 | Differential lipid analysis: visualization of significantly altered lipid subclasses and molecules across comparisons. A Sankey diagram illustrating the distribution of the top 10 lipid subclasses with the highest abundance among significantly altered lipid species across ND, KO1, and KO6 groups. B Heatmap of the top 50 significantly altered lipid molecules, ranked by increasing *p* value. The color scale represents the relative abundance of lipid molecules after normalization of peak areas across samples, with red indicating higher abundance and blue indicating lower abundance. Volcano plots showing differential lipid molecules in the

comparisons of KO1 versus ND (C), KO6 versus ND (D), and KO6 versus KO1 (E). F Venn diagram showing the overlap of significantly altered lipid molecules among the three pairwise comparisons. ADGGA acyl diacylglycerol glucuronide, CE cholesterol ester, DGCC diacylglycerol-3-O-carboxyhydroxymethylcholine, FA fatty acyl, LPC lysophosphatidylcholine, MGDG monogalactosyldiacylglycerol, PC phosphatidylcholine, SE Sitosterol ester, SHexCer sulfolipid, TG triacylglycerol; FC fold change.

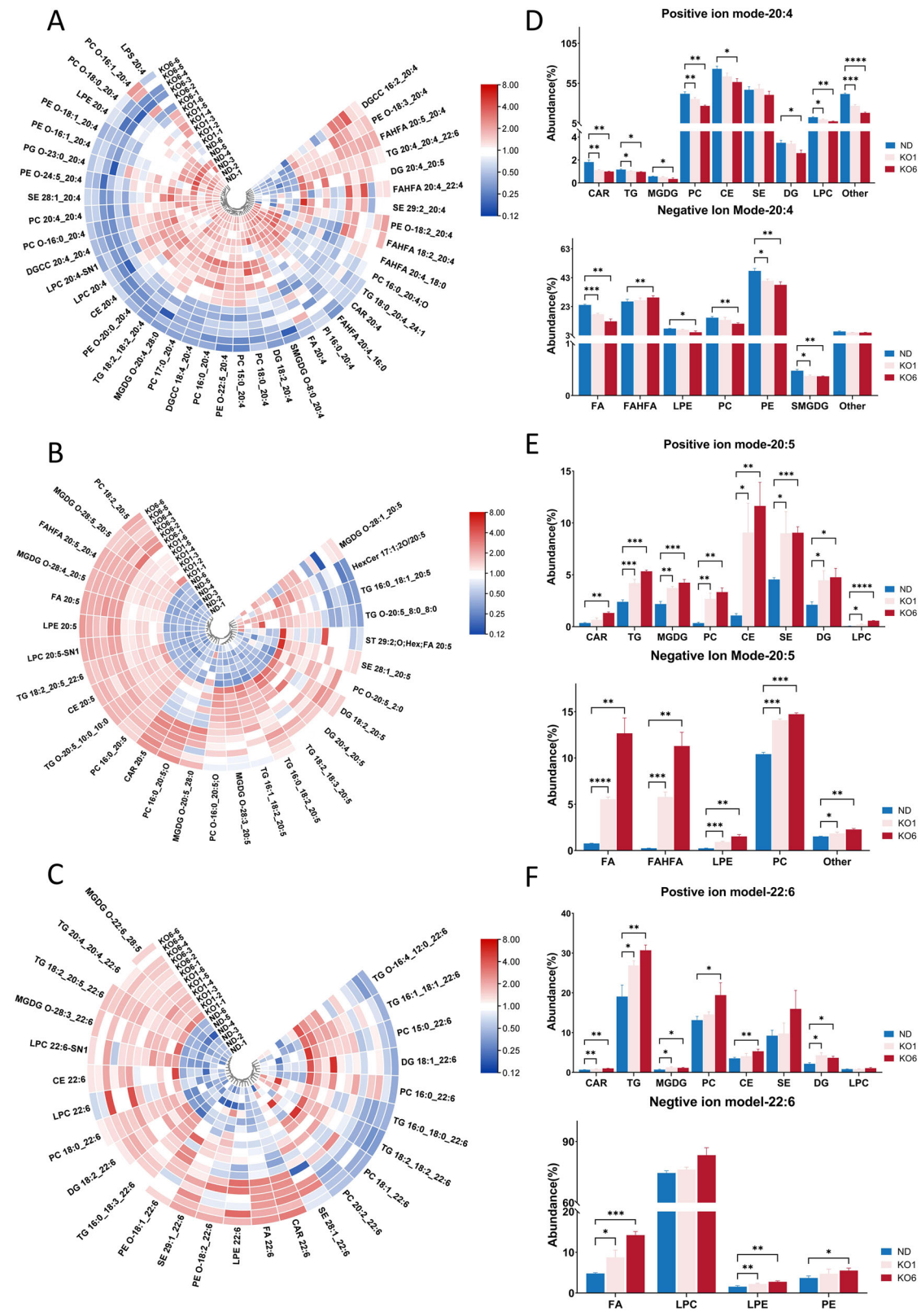


Fig. 3 | Distribution patterns of ARA-, EPA-, and DHA-containing lipid molecules and subclasses across groups and ionization modes. The heatmaps showing the relative abundance of lipid molecules containing ARA (A), EPA (B), and DHHA (C). Colors represent the relative abundance after normalization of peak areas, with red indicating higher abundance and blue indicating lower abundance. Relative

abundance of lipid subclasses containing ARA (D), EPA (E), and DHA (F) detected under positive and negative ionization modes. Statistical significance was determined using one-way ANOVA, with Tukey's HSD applied for post hoc test. Significance levels are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

contain ARA, EPA, and DHA, respectively. We analyzed the lipid subclasses containing these three fatty acids under both positive and negative ion modes and conducted normalization. The findings were displayed in Fig. 3D–F. Overall, compared to the ND group, dietary supplementation with krill oil for 1 week resulted in a decreasing trend in ARA levels across most lipid subclasses. After 6 weeks of krill oil supplementation, a significant reduction in ARA levels was observed in most lipid subclasses, such as TG, PC, and LPC in the positive ion mode, as well as FFA and PE, etc. in the negative ion mode. In contrast, compared to the ND group, dietary supplementation with krill oil resulted in higher levels of EPA and DHA across most lipid subclasses in rats. This increase was more pronounced after 6 weeks of krill oil supplementation compared to 1 week, with notable changes seen in CAR, TG, and MGDG in the positive ion mode, and FFA and LPE, etc. in the negative ion mode. This finding was consistent with research by sung et al.²⁰, which showed that dietary supplementation with ω-3 PUFAs can increase the levels of EPA and DHA in the body.

Differences in oxylipin profiles in rat after krill oil dietary intake

We evaluated the levels of three essential PUFAs (ARA, EPA, and DHA) and their derivatives in rats using targeted lipidomics. The oxylipins were identified based on published criteria^{24,25}, which required matching retention times (± 0.05 min), signal-to-noise ratio (S/N) ≥ 3 for LOD, peak height $\geq 5\times$ baseline, fragmentation patterns, and the presence of at least six characteristic and diagnostic ions. The importance of chromatographic separation for oxylipins due to the presence of the numerous stereoisomers and regioisomers with varying hydroxyl group positions (*R*, *S*) and conjugated double bond configurations (*E*, *Z*). These isomers typically display identical fragmentation patterns and similar chromatographic behavior²⁵. For this study, a high-performance C18 reverse-phase chromatography column was employed to achieve effective separation. Currently, the analysis of SPMs and other oxylipins typically relies on ESI and the use of MRM methodologies. This approach employs established methods with predefined transitions for each mediator of interest, monitoring both the precursor ion and a characteristic product ion for each compound, which can help to avoid cross-talk and short run times. Table S2 provided details on the retention time, the precursor ion (Q1) and a characteristic product ion (Q3) for the standard products. The retention times for the three precursors, ARA, EPA, and DHA, were 11.96, 11.57, and 11.84 min, respectively. The retention times for all the oxylipins detected ranged from 7.69 to 10.83 min. The retention time of deuterated internal standards was displayed in Table S3. The representative MS/MS spectra for the oxylipins were presented in Fig. S4. For instance, the MS/MS spectrum of LXA₄ displayed characteristic fragment ions, including *m/z* 351 ([M-H]⁻), 333 ([M-H-H₂O]⁻), 307 ([M-H-CO₂]⁻), 289 ([M-H-H₂O-CO₂]⁻), 217 ([235-H₂O]⁻), 199 ([235-2H₂O]⁻), and 189 ([251-H₂O-CO₂]⁻). A total of 33 oxylipins were identified and quantified, comprising 12 derivatives of ARA, 8 derivatives of EPA, and 13 derivatives of DHA. The quantification of these lipid mediators was performed using a deuterium labeled stable isotope as an internal standard, alongside an external calibration curve. The calibration curves were generated based on the area ratio of analyte to internal standard versus analyte concentration. Linear regression with a weighting factor of 1/*X* was performed for each standard and the linearity and the accuracy of the detection were evaluated. The calibration curves of the oxylipins were listed in Table S2. All calibration curves had a coefficient of determination (*R*²) value exceeding 0.9964, indicating that they were accurate and reliable. Three fatty acids and 33 of their metabolites were quantified and the results were displayed in Fig. 4.

ARA derived oxylipins

Figure 4A displayed a simplified schematic representation of the ARA metabolic pathway. ARA is derived via 5-LOX to form 5-hydroxyeicosatetraenoic acid (5-HpETE), which is subsequently converted to LTs (e.g., LTB₄). Additionally, ARA is oxygenated via COXs to produce PGH₂, which is further converted into PGs (PGD₂, PGE₂, and PGF₂) and TXB₂. These mediators contribute to hallmark inflammatory

processes, including enhancing vascular permeability, recruiting leukocytes, promoting thrombosis, and upregulating the expression of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)²⁹. In another pathway, ARA is converted by 5-LOX and 15-LOX to produce 5-HpETE and 15-HpETE, respectively, which are then transformed into anti-inflammatory LXs (LXA₄ and LXB₄)³⁰. Among the derivatives of ARA, we identified 12 lipid mediators. In ND group, TXB₂ was the most abundant oxylipins with 591.50 \pm 30.90 ng/mL, followed by 12-HETE (361.34 \pm 49.92 ng/mL) and LXA₄ (120.42 \pm 15.82 pg/mL). As illustrated in Fig. 4A, a decreasing trend in the levels of ARA and most ARA derivatives after krill oil supplementation. Specifically, a significant reduction was observed after 6 weeks, with a 64.5% decrease in ARA concentration (0.35-fold reduction) in the serum compared to the normal diet. For the ARA derivatives, after 1 week of krill oil supplementation, we observed a significant decrease in the levels of six derivatives: 5-HETE, 12-HETE, TXB₂, PGD₂, PGE₂, and LTB₄. Five other derivatives showed no significant change, while only LXA₄ levels increased significantly (3.84-fold). After 6 weeks of supplementation, significant changes were noted in all derivatives except for PGF_{2 α} and LXB₄. Ten compounds exhibited notable decreases, while LXA₄ levels increased significantly, rising from 120.42 \pm 15.82 pg/mL to 758.70 \pm 45.57 pg/mL. This result aligned with the study by Gart et al., where the levels of ARA and most ARA-derived oxylipins decreased after krill oil dietary intake³¹.

EPA derived oxylipins

In the simplified metabolic pathway of EPA (Fig. 4B), EPA is initially converted into 18-hydroperoxide intermediate (18-HpEPE) by COX-2 or CYP450 enzymes, which intermediate is then transformed into 18R-hydroxyeicosapentaenoic acid (18R-HEPE). This intermediate can be further biosynthesized into RvE1 and RvE2 by 5-LOX, or into RvE3 in the presence of 15-LOX. Additionally, EPA can be metabolized by 15-LOX to produce 15-HpEPE, which is then converted into 15-HEPE. This compound will be finally transformed via 5-LOX to RvE4 and LXA₅^{32,33}. Furthermore, EPA is converted to 5-HEPE and 12-HEPE by 5-LOX and 12-LOX enzymes, respectively³⁴. In the derivatives of EPA, we identified four SPMs—namely RvE1, RvE2, RvE4, and LXA₅—along with four other lipid mediators. In the ND group, the concentrations of these four SPMs varied from 6.38 \pm 0.50 pg/mL (RvE2) to 155.71 \pm 9.08 pg/mL (LXA₅), whereas the four HEPEs were notably higher with the concentrations range from 1.05 \pm 0.11 pg/mL (15S-HEPE) to 227.96 \pm 14.34 pg/mL (18-HEPE). As depicted in Fig. 4B, the serum levels of EPA in rats increased significantly from 0.69 \pm 0.04 μ g/mL to 1.88 \pm 0.14 μ g/mL after 1 week of dietary supplementation with krill oil. Over 6 weeks supplementation, its level rose further to 2.73 \pm 0.17 μ g/mL (3.96-fold increase). Following krill oil supplementation, the levels of EPA-derived oxylipins generally increased. The levels of all derivatives except for 5-HEPE, 15-HEPE, and LXA₅ were significantly increased after 1 week of supplementation, whereas the content of all eight derivatives exhibited notable increases after 6 weeks. Among them, RvE1 showed the highest increase, rising by 14.27-fold, followed by 12-HEPE, which increased by 4.61-fold.

DHA derived oxylipins

DHA is processed by 15-LOX to produce a 17-hydroperoxide product, 17S-HpDHA, which is further converted into RvD1-D6 in the presence of 5-LOX. In addition to being a precursor for D-series Rvs, the 17S HpDHA intermediate also serves as a precursor for the 17S epoxy intermediate, which is then hydrolyzed to produce PD1. Moreover, DHA is converted to 14S HpDHA intermediate in the presence of 15-LOX, which is then hydrolyzed to produce MaR1 and MaR2^{35,36}. In this study, we identified multiple DHA derivatives in rats. These included eight SPMs—RvD1 to RvD5, MaR1, MaR2, and PD1—with concentrations varying from 7.13 \pm 0.79 pg/mL (PD1) to 54.77 \pm 4.01 pg/mL (MaR1) in the ND group. Additionally, we detected five other lipid mediators (HDHAs), whose concentrations ranged from 32.35 \pm 2.99 pg/mL (7-HDHA) to 42.18 \pm 5.61 ng/mL (14-HDHA). We found that the serum levels of DHA

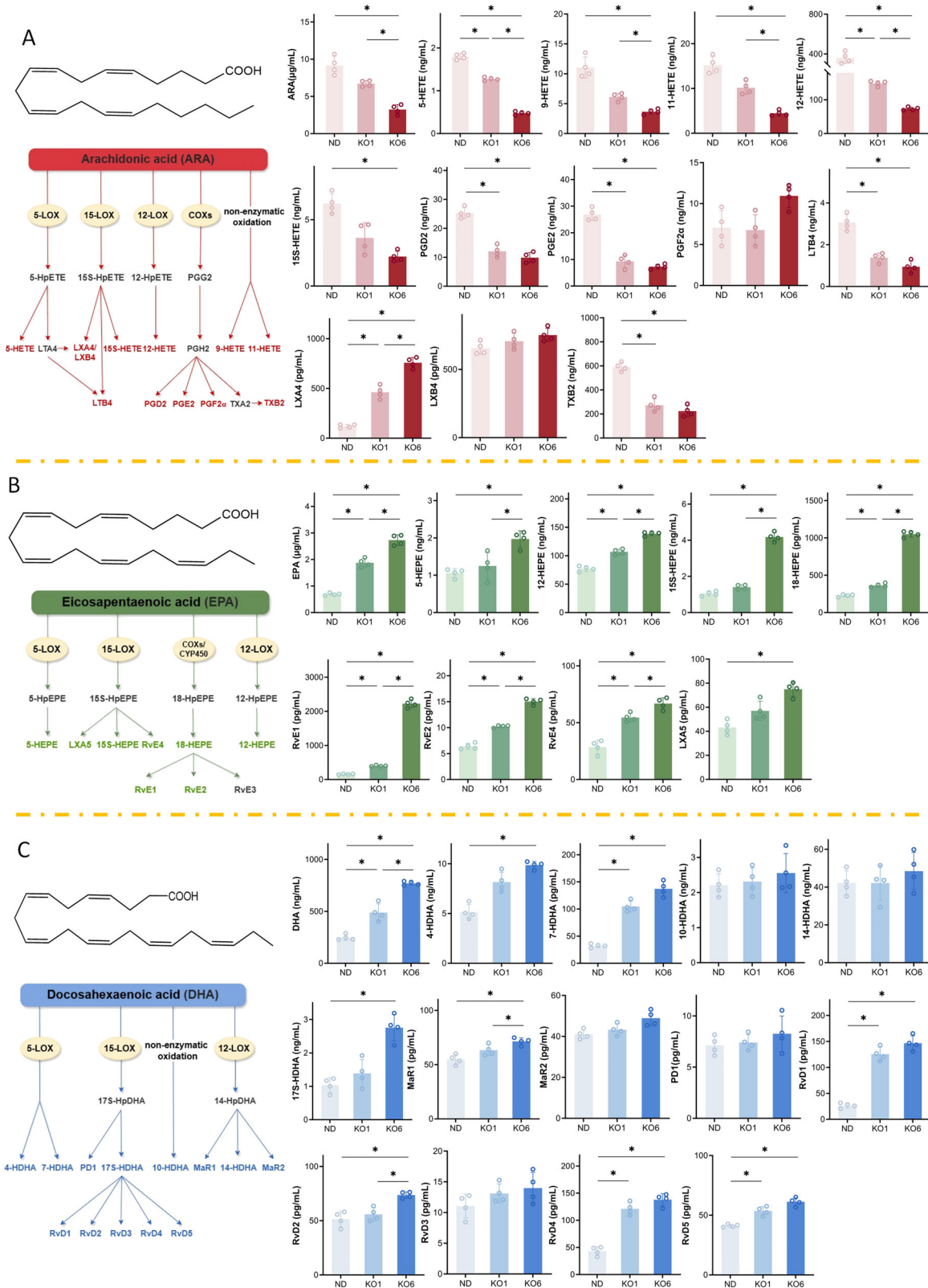


Fig. 4 | Changes in serum oxylipin levels after 1 week and 6 weeks of KO diet supplementation. The simplified schematic representation of the metabolic pathway and the concentration of the derivatives of ARA (A), EPA (B) and DHA (C).

Statistical significance was determined using one-way ANOVA, with Tukey’s HSD applied for post hoc test. Significance levels are indicated as $p < 0.05$ (*).

followed a similar pattern to EPA after dietary supplementation with krill oil. Specifically, there was a significant increase in DHA levels, rising from $248.54 \pm 24.35 \mu\text{g/mL}$ to $488.65 \pm 72.71 \mu\text{g/mL}$ after 1 week of dietary supplementation with krill oil. The concentration continued to increase to

$770.47 \pm 16.26 \mu\text{g/mL}$ after 6 weeks, representing a 3.10-fold rise. As illustrated in Fig. 4C, following 1 week of supplementation, the levels of four compounds—7-HDHA, RvD1, RvD4, and RvD5—were significantly elevated. After 6 weeks, there was a notable increase in eight derivatives,

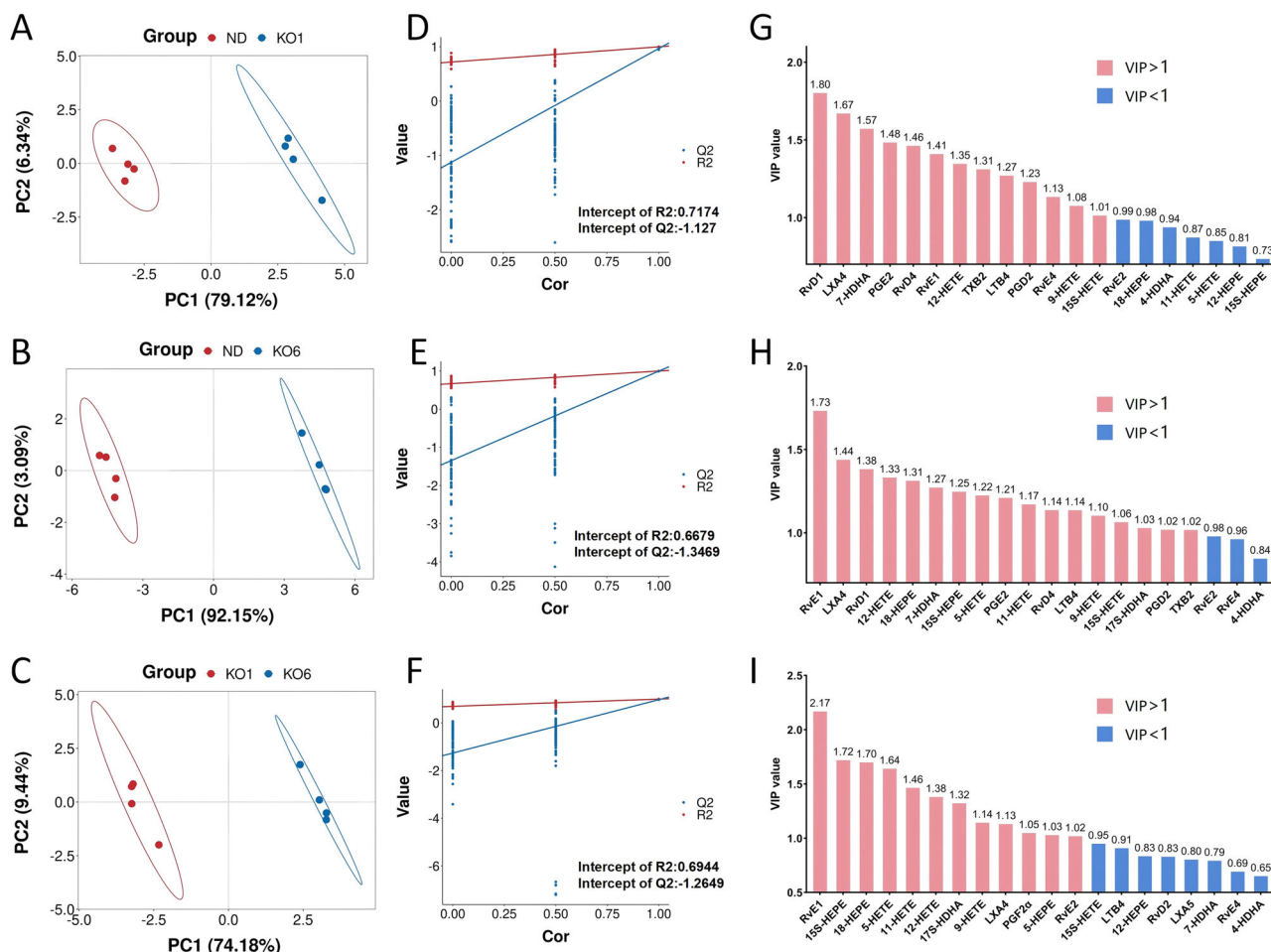


Fig. 5 | The PLS-DA models built based on targeted oxylipin profiles. PLS-DA score plots showing the discrimination of oxylipin profiles between KO1 versus ND (A), KO6 and ND (B), and KO6 and KO1 (C). D–F The permutation test (200 iterations)

used to validate the robustness and to assess potential overfitting of the corresponding PLS-DA models. G–I Variable importance in projection (VIP) plots highlighting the oxylipin species contributing most to group discrimination in each comparison.

including the aforementioned four compounds and additionally 4-HDHA, 17S-HDHA, MaR1, and RvD2. Meanwhile, the levels of the remaining five derivatives experienced minor elevations that were not statistically significant ($p > 0.05$), both in the short and long term, due to the krill oil dietary supplementation.

Correlation analysis among ARA, EPA, and DHA-derived oxylipins

To further investigate the changes in oxylipin levels in rats’ serum following krill oil supplementation, three PLS-DA models based on ND versus KO1, ND versus KO6, and KO1 versus KO6 were developed. The score plots (Fig. 5A–C) from these PLS-DA analyses observed that samples were distributed on both sides of the X-axis, indicating significant differences in oxylipin levels between each pair of groups. The permutation test, conducted with 200 iterations was displayed in Fig. 5D–F. The intercepts of R^2 values ranged from 0.6679 to 0.7174, while the intercepts of Q^2 values ranged from -1.3469 to -1.127 . These results indicated that the models were robust, with a low risk of overfitting. A variable importance for the projection (VIP) plot was built to identify the significant difference molecules responsible for the alterations in the levels of oxylipins after short and long-term krill oil dietary intake. The molecules with VIP values exceeding 1 were considered significant difference molecules³⁷. Across the three comparison groups, we identified 13, 17, and 12 significantly different molecules, respectively (Fig. 5G–I). Specifically, in the comparisons of KO1 versus ND, the top 5 differential molecules were RvD1, LXA₄, 7-HDHA, PGE₂ and RvD4; in the KO6 versus ND comparison, the leading molecules were RvE1,

LXA₄, RvD1, 12-HETE, and 18-HEPE; and in the comparison of KO1 versus KO6, RvE1, 15S-HEPE, 18-HEPE, 5-HETE and 11-HETE emerged as the key differential molecules. Interestingly, the significant difference lipid mediators identified in the comparisons between ND and KO1, as well as between ND and KO6, exhibited a high level of overlap. This indicated that both short-term and long-term dietary supplementation with krill oil have a consistent effect on regulating most oxylipins levels in rat. The correlation analysis was performed between ARA derivatives and EPA/DHA derivatives, with the resulting network diagram shown in Fig. S5. We observed that the levels of AA and most of its derivatives were negatively correlated with the levels of EPA, DHA and their respective derivatives. In contrast, the situation for LXA₄ was exactly the opposite. It was positively correlated with most EPA/DHA derivatives, including HEPEs, RvEs, and RvDs.

Discussion

In this study, we employed non-targeted lipidomics to comprehensively analyze the effects of daily dietary supplementation with krill oil on lipid metabolism in rat serum. A notable alteration in the lipid composition of rat serum was observed after dietary supplementation with krill oil. The most abundant differential subclasses were TG, PC, and LPC, which are central to systemic energy handling and membrane-associated signaling. TGs are the predominant neutral lipid storage form and a major reservoir of metabolic energy³⁸. PC is a principal membrane phospholipid and, in many mammalian cell types, represents 40–50% of cellular phospholipids; beyond structural roles, PC turnover supplies bioactive lipid intermediates that participate in signal transduction³⁹. LPC is generated primarily via

PLA2-mediated hydrolysis of PC and can act as a context-dependent immunomodulatory lipid mediator; for instance, LPC can engage TLR-associated signaling in macrophages and modulate downstream inflammatory responses⁴⁰. Notably, the observed shift in the lipid profile—lower abundance of 20:4-containing species and higher abundance of 20:5/22:6-containing species—reflects both the competitive metabolism between ω -3 and ω -6 fatty acids and the enrichment of krill oil-derived ω -3 fatty acids (typically bound to phospholipids) in the circulating lipid pools. In a randomized, placebo-controlled crossover study in healthy volunteers, krill oil increased plasma and RBC ω -3 PUFA (including EPA/DHA) and reduced the ω -6: ω -3 ratio relative to fish oil³. At the same time, we acknowledge literature showing that a consistent “bioavailability advantage” for krill oil is not universal and may disappear when dose and formulation are carefully matched or when alternative endpoints are considered^{5,6}. Additionally, some lipid molecules containing EPA and DHA were only increased after 1 week and not at 6 weeks of KO supplementation, such as TG O-16:4_12:0_22:6, TG 16:1_18:1_22:6 and DG 18:1_22:6, etc. An early rise in circulating EPA/DHA-containing lipid species at 1 week may reflect rapid absorption and lipoprotein transport, and may subsequently reach a steady state (plateau) with smaller marginal changes in serum despite continued dosing. By 6 weeks, a larger portion of supplemented EPA/DHA may be retained in cellular/tissue phospholipid membranes rather than remaining elevated in the circulating (serum) lipid pool, leading to attenuated differences in serum lipid molecular species^{6,41}. The elevated availability of EPA and DHA substrates, as evidenced by our lipidomics analysis, is likely to enhance the biosynthesis of SPMs through enzymatic conversion pathways mediated by lipoxygenases. This lipidomic remodeling have profound physiological significance, which are supporting its potential cardioprotective and pleiotropic benefits through improved lipid homeostasis and attenuated inflammatory signaling⁴².

Multiple method-development and application studies have demonstrated that many SPMs in healthy plasma/serum are frequently below the lower limit of quantification (LLOQ), and that detectability depends strongly on matrix choice, sample handling, and rigorous validation^{27,28}. For instance, highly optimized LC–MS/MS method have reported that circulating SPM levels in healthy biological matrices can be extremely low or not quantifiable, while inflammatory contexts may increase detectability in some settings²⁵. To date, some studies have reported the detection of various SPMs in biological samples; however, the validity of some of these findings has been questioned²⁸ and the levels of oxylipins also varied across different studies. Notably, despite these challenges, our method enabled the quantification of several SPMs, including RvE1, RvD1, and MaR1, etc. Our analytical method shares similarities with the LC–MS-based method developed by Fu et al. They successfully quantified multiple SPMs in mouse plasma/liver and human plasma samples, including RvE1, RvE2, LXA₄, LXA₅, RvD1 and RvD2, etc⁴³. In contrast to Fu et al., our study employed rat serum rather than plasma as the biological matrix. Moreover, we used a substantially larger sample volume (500 μ L of serum), which may have increased analyte enrichment and improved detection sensitivity. These differences in matrix type and sample volume may partially explain the discrepancies observed in SPM detectability between studies.

Targeted oxylipins analysis revealed that krill oil dietary supplements significantly reduced the levels of most ARA-derived oxylipins (such as HETEs, TXB₂, PGs, LTB₄), while markedly elevating LXA₄ levels in a time-dependent manner. Mounting evidence has substantiated that ARA and its metabolites play pivotal roles in both organ development and the pathogenesis of various diseases, encompassing cardiovascular, renal, hepatic, and skeletal development, as well as metabolic disorders (e.g., diabetes mellitus, obesity) and malignant tumors⁴⁴. LXs, a class of ARA-derived metabolites, exhibit anti-inflammatory and immunomodulatory activities. Zhu et al. demonstrated that administration of LXA₄ enhanced the restoration of neurological function and tissue integrity following brain injury in rat, while maintaining the integrity of the blood–brain barrier and suppressing neuroinflammatory responses⁴⁵. The sustained reduction in pro-inflammatory oxylipins, coupled with a progressive elevation in LXA₄ levels following krill

oil supplementation, indicates a temporal reconfiguration of the oxylipin profile favoring resolution pathways and anti-inflammatory responses. In parallel, krill oil markedly elevated multiple EPA- and DHA-derived metabolites, including 12-HEPE, 18-HEPE, RvEs, 7-HDHA, RvDs and MaR1. This result aligned with several intervention studies reporting that increasing dietary EPA/DHA shifts systemic oxylipin patterns toward ω -3-derived products and away from ω -6 (ARA)-derived products. Ostermann et al. reported a large randomized controlled trial in which plasma oxylipins (quantified by targeted LC–MS) showed a clear linear dose–response increase in EPA- and DHA-derived oxylipins over 3 and 12 months of EPA + DHA intake. They revealed that the relative increase in concentrations of EPA-derived oxylipins was substantially greater than that of oxylipins derived from DHA⁴⁶. This trend was also observed in our research. In a 12 weeks intervention, Schebb et al. observed that EPA + DHA ethyl esters increased free and total EPA-derived hydroxy/epoxy/dihydroxy oxylipins (70–150%) while total AA-derived metabolites decreased on average (30%), indicating that even in a relatively healthy cohort the directionality of change can favor an AA-to-EPA shift⁴⁷. Taken together, these findings suggest that ω -3 supplementation reshapes the oxylipin profile primarily via substrate remodeling^{48,49}, which is consistent with our observed suppression of ARA-derived mediators and induction of EPA/DHA-derived mediator families. Our finding that krill oil supplementation elevated EPA/DHA-derived mediators, such as 18-HEPE and 7-HDHA, is also consistent with reports that ω -3 intake increases not only classical LOX products but also CYP-derived epoxy metabolites and SPM precursors in healthy humans. In a clinical intervention, ω -3 supplementation resulted in dose-dependent increases in EPA/DHA-derived epoxy metabolites, and 18-HEPE rose markedly in supplemented groups, while AA-derived epoxy metabolites (EETs) were comparatively unchanged¹³. The trends reported in the literature are broadly consistent with the directional changes observed in our study (increased EPA/DHA mediator families). At the same time, these reports highlight that the magnitude and breadth of changes can vary with formulation, dose, and duration^{13,50}. Moreover, we found that the relationship between mono-hydroxylated precursors and terminal SPMs is not necessarily linear. For example, 17-HDHA are commonly treated as precursors for D-series-Resolvins⁵¹, yet precursor accumulation does not guarantee proportional increases in a specific derived mediator (RvD3). This discordance can arise from pathway branching, enzyme competition and transcellular conversion requirements.

Importantly, not all literatures fully agree on the extent of ARA-derived oxylipins suppression. In this study, the majority of ARA-derived oxylipins showed a stronger reduction than some previous ω -3 interventions. The analytical compartment (serum versus plasma), and whether oxylipins are measured as free versus total (free + esterified) pools, can influence the results. Schebb et al. showed that total oxylipins can be orders of magnitude higher than free oxylipins for certain classes, yet the ratio of free/esterified remained stable after supplementation⁴⁷. Consequently, determining whether the free or total lipid pool is measured may modulate the results. Furthermore, even when fatty acid intake remains consistent, significant interindividual variations may still occur. In a controlled supplement study, despite similar shifts in erythrocyte EPA/DHA and AA, divergence in serum oxylipin responses was observed⁵². Furthermore, the present study was conducted exclusively in male rats. Given that biological sex can shape lipid and oxylipin landscapes and modulate dietary responsiveness, future krill oil intervention studies should include both sexes and be designed to delineate sex-specific lipidomic and oxylipin/SPM responses to krill oil supplementation.

Notably, the time-dependent elevation of SPMs holds significant physiological implications. As pivotal regulators of inflammatory resolution, SPMs exert multifaceted protective effects: they mitigate the release of proinflammatory cytokines, facilitate phagocytic clearance of apoptotic cells, and reestablish tissue homeostasis without compromising host defense⁵³. Clinical evidence has consistently associated elevated levels of SPMs with ameliorated disease progression and enhanced clinical outcomes in chronic inflammatory disorders, including atherosclerosis and

rheumatoid arthritis. Studies on human carotid atherosclerotic plaques have a marked imbalance in SPMs to pro-inflammatory lipid mediators—such as leukotrienes—within vulnerable plaque regions. Restoration of SPM levels has been associated with delayed atherosclerosis progression and a transition toward a more stable plaque phenotype⁵⁴. In addition, Barden et al. demonstrated that synthesis of SPMs at inflammatory foci constitutes a relevant mechanistic through which ω -3 PUFAs mitigate the clinical manifestations of arthritis⁵⁵. These findings underscore the potential of long-term krill oil supplementation as a nutritional strategy to support resolution pathways and mitigate inflammatory burden.

This study incorporated non-targeted and targeted lipidomics approaches to detect the comprehensive lipid profile and oxylipins levels following short-term (1 week) and long-term (6 weeks) krill oil supplementation. Specifically, after 1 week of supplementing with krill oil, the levels of most 20:4-containing lipid molecules decreased compared to the ND group, while those with 20:5 and 22:6 increased. These trends became more significant after 6 weeks of supplementation. In the targeted lipidomics analysis, we identified and quantified a total of 33 oxylipins consisting of 12 derivatives of ARA, 8 derivatives of EPA, and 13 derivatives of DHA. The results indicated that krill oil supplementation reduced pro-inflammatory oxylipins from ARA and boosted most EPA- and DHA-derived oxylipins, especially SPMs, resulting in significant alterations in the overall oxylipin profile in rat serum. In conclusion, dietary supplementation with krill oil can elevate SPMs levels and decrease pro-inflammatory mediator content, helping the body achieve a balanced state of homeostasis. This study provides new insights into the biochemical pathways through which krill oil exerts its beneficial effects, indicating its potential as a dietary intervention for managing chronic inflammation and enhancing overall health.

Methods

Materials and reagents

Krill oil was provided by Liaoyu Fishery Group Co., Ltd. (Liaoning, China). Methanol, acetonitrile, ethyl acetate, isopropanol, and n-hexane, all of LC/MS grade, were procured from Merck (Darmstadt, Germany). Formic acid, acetic acid, and disodium hydrogen phosphate were of analytical grade and provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). High-purity water with a resistivity of 18.2 M Ω cm was sourced from the Millipore Milli-Q water system (Bedford, MA, USA).

Animals and treatments

Thirty specific pathogen-free, male, Sprague-Dawley rats (150–200 g, 6–8 weeks old) were purchased from Shanghai BK Co., Ltd. and housed at the research center of Zhejiang Chinese Medical University. Rats were acclimatized to laboratory conditions for at least 1 week prior to experiments (five rats per cage). Krill oil was provided by Liaoyu Antarctic Krill Technology Development Co., Ltd. (Liaoning, China).

All animal experiments approved by the Ethics Committee of Zhejiang Chinese Medical University Laboratory Animal Research Center (Permission Number: IACUC-20220829-14), and were strictly in accordance with the local Guidelines for Laboratory Animal Care of Animal Experimental Center, Zhejiang Chinese Medical University. After 1 week of acclimatization, the rats were randomly divided into three groups ($n = 10$ in each group): ND, KO1, and KO6. Rats were fed a standard SPF-grade chow diet (Beijing Keao Xieli Feed Co., Ltd., Beijing, China). The general composition of the normal diet was shown in Table S4. Rats in the KO1 and KO6 groups received krill oil by oral gavage at a daily dose of 1 g/kg. The fatty acid composition of the krill oil is presented in Table S5. At the end of 1 week, rats in the ND and KO1 groups were fasted for 12 h, anesthetized, and euthanized. Rats in the KO6 group underwent the same procedure at the end of 6 week.

Blood samples collection

For terminal blood collection, rats were anesthetized using sodium pentobarbital administered intraperitoneally (50 mg/kg). Adequate depth of

anesthesia (deep surgical anesthesia/unconsciousness) was verified prior to incision by loss of righting reflex and the absence of pedal withdrawal (toe pinch) and corneal reflex. While under deep anesthesia, terminal blood sampling was performed via abdominal aortic puncture. Immediately after blood collection, animals were euthanized by anesthetic overdose (additional pentobarbital to achieve a total dose of 200 mg/kg, i.p.). Death was confirmed by cessation of respiration and absence of cardiac activity and a secondary physical method (bilateral thoracotomy) was performed to ensure death. The serum samples were incubated at room temperature for 30 min, then centrifuged at 3000 rpm for 15 min at 4 °C. The collected serum samples were promptly frozen using dry ice and stored at –80 °C until used.

Non-targeted lipidomics analysis

The collected serum sample was thawed on ice, and 20 μ L of the sample was taken. This was then mixed with 120 μ L of precooled lipid extraction buffer (isopropanol/acetonitrile/water, 2/1/1, v/v/v), vortexed for 1 min, and incubated at room temperature for 10 min. The extraction mixture was then stored at –20 °C overnight. After centrifugation (4000 \times g, 20 min), the supernatant was collected. The samples were stored at –80 °C before LC-MS/MS analysis.

Chromatographic separation was performed using an ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a Kinetex UPLC C18 column (100 mm \times 2.1 mm, 100 A, phenomenex, UK). The column temperature was maintained at 55 °C. The mobile phase comprised solvent A (isopropanol/acetonitrile, 9/1, v/v, with 0.1% formic acid) and solvent B (acetonitrile/water, 6/4, v/v, with 0.1% formic acid). The gradient elution conditions were as follows: 0–0.4 min, 30% A; 0.4–1 min, 30% to 45% A; 1–3 min, 45% to 60% A; 3.5–5 min, 60% to 75% A; 5–7 min, 75% to 90% A; 7–8.5 min, 90% to 100% A; 8.5–8.6 min, 100% A; 8.6–8.61 min, 100% to 30% A. The flow rate was set to 0.3 mL/min.

The mass analysis was performed on a high-resolution tandem mass spectrometer TripleTOF6600 (SCIEX, Framingham, MA, USA). The mass scan range was from 60 to 1200 Da in both positive and negative ion modes, and the data were acquired using Information-Dependent Acquisition (IDA) mode. The instrument parameters were as follows: curtain gas was set to 30 psi; ion source gas 1 and ion source gas 2 were both set to 60 psi; the interface heater temperature was maintained at 650 °C; and the ionspray voltages were 5000 V for positive ion mode and –4500 V for negative ion mode.

Targeted oxylipins analysis

Oxylipins extraction: the oxylipins of the serum samples were extracted using solid phase extraction (SPE)⁴³. Briefly, 500 μ L of serum was spiked with 5 μ L of a deuterium-labeled internal standard solution (20 ng/mL d-oxylipins, 20 μ g/mL ARA-d8, EPA-d5 and DHA-d5) and an antioxidant solution containing 0.2 mg/mL butylated hydroxytoluene (BHT), 100 μ M indomethacin, and 100 μ M soluble epoxide hydrolase inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) in methanol). Detailed information regarding the standard produces can be found in Table S6. Then, added 1 mL ethyl acetate/methanol (5:1, v/v) to mixture and ultrasound-assisted extraction for 15 min. After centrifugation (12,000 r/min for 2 min), the supernatant was collected and the lower phase was further subjected to two re-extractions following the same method. The collected supernatants were combined. Furthermore, the collected supernatants were combined and evaporated to dryness in a vacuum concentrator at 25 °C and 1 mbar for approximately 90 min (Christ, Osterode, Germany). The sample extracts were reconstituted in 3 mL 0.1 M disodium hydrogen phosphate adjusted to pH 6.0 with acetic acid in water/methanol (95/5, v/v) and submitted to solid-phase extraction using SPE column (Bond Elut Certify II, 200 mg, 3 mL; Agilent, Waldbronn, Germany).

The SPE cartridge was preconditioned with one column volume of each of the following: ethyl acetate/hexane (75/25, v/v, with 1% acetic acid), methanol, and 0.1 M disodium hydrogen phosphate buffer adjusted to pH 6.0 with acetic acid in a water/methanol (95/5, v/v). The sample was loaded

onto the preconditioned cartridge at a flow rate of approximately 0.5 mL/min. After complete loading, the cartridge was washed with 3 mL water and 3 mL water/methanol (50/50, v/v), and then dried under a nitrogen stream at high pressure for 1 min. The analytes were eluted with 2 mL of ethyl acetate/n-hexane (75/25, v/v, with 1% acetic acid) into tubes containing 6 μ L of a 30% glycerol solution in methanol. The eluate was then evaporated using a vacuum concentrator (30 °C and 1 mbar for approximately 120 min) until only the glycerol plug remained. The residue is redissolved in 100 μ L methanol and stored at -80 °C before LC-MS/MS analysis.

LC-MS/MS: chromatographic separation was performed on ExionLC Series UHPLC (Applied Biosystems SCIEX, Foster City, USA) equipped with an ACQUITY UPLC C18 reversed phase column (100 mm \times 2.1 mm; 1.7 μ m). The column was washed and readjusted to the initial conditions before each run and the column temperature was kept 30 °C. The mobile phase consisted of solvent A (0.02% aqueous acetic acid) and solvent B (acetonitrile). The gradient elution conditions were as follows: 0–5 min, 20% B; 5–11 min, 20% to 95% B; 11–15 min, 95% B; 15–15.1 min, 95% to 98% B; 15.1–25 min, 98% B; 25–25.1 min, 98% to 20% B; 25.1–30 min, 20% B, at a flow rate of 0.4 mL/min.

The mass analysis was performed on a Qtrap 6500 mass spectrometer (Applied Biosystems SCIEX, Darmstadt, Germany) equipped with an ESI source. The analysis was conducted in negative ion mode, and ions were identified and quantified using multiple reaction monitoring (MRM). Compounds were quantified using stable internal standards. The operating conditions were as follows: the temperature was 600 °C; the ion spray voltage was -2.5 kV; the declustering potential was -60 eV, and the entrance potential was -7 eV; the curtain gas, ion source gas 1 (nitrogen drying gas), and ion source gas 2 (nebulizer gas) were all set to 35 psi. The data collection was performed using Analyst 1.6.2 software, and quantification was carried out using MultiQuant software (SCIEX, Framingham, MA, USA).

Statistical analysis

Microsoft Excel software was employed to calculate the mean value and standard deviation of the samples. Multivariate statistical analysis was performed using the MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>) and OmicStudio tools (<https://www.omicstudio.cn/tool>). Statistical analyses were performed using SPSS version 23.0 (SPSS Inc., Chicago, IL). Differences among experimental groups were assessed by one-way analysis of variance (ANOVA), with Tukey's HSD applied for post hoc test. The Benjamini–Hochberg procedure was adopted for false discovery rate (FDR) correction to account for the multiple testing problem. The adjusted $p < 0.05$ was considered statistically significant. The heatmap were generated using TBtools.

Data availability

All data supporting the findings of this study are available in the paper and Supplementary Information. Data will be made available upon request.

Received: 7 December 2025; Accepted: 20 January 2026;

Published online: 10 February 2026

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Acknowledgements

This study was funded by the Zhejiang Provincial Natural Science Foundation of China (MS25H020020); Zhejiang Provincial Public Welfare Technology Research Project (grant number: LTGY24H020004); Zhejiang Medical Health Science and Technology Plan (grant number: 2023XY006 and 2023KY091); Zhejiang Medical Association Clinical Medical Research Special Project (grant number: 2023ZYC-Z34); Zhejiang Pharmaceutical Society Hospital Pharmacy Special Research Funding Project (grant number: 2023ZY34); Hangzhou Medical Health Technology Project (grant number: B20231798 and B20252352); Ningbo Young Scientific and Technological Innovation Leading Talent Program (grant number: 2024QL027); Linping District Agricultural and Social Development Research Projects (grant number: 2022015); Linping Medical Health Technology Project (grant number: LPWJ2022-01-03, LPWJ2023-02-38, and LPWJ2023-01-16).

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41538-026-00727-5>.

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