

Anti-arthritic effects of polyunsaturated fatty acid-rich supplementation combined with selective soluble epoxide hydrolase inhibitors in a collagen-induced arthritis mouse model

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ABSTRACT

Objectives: Polyunsaturated fatty acids (PUFAs) are healthy fats that contain a double carbon bond. They are made up of omega (ω)-3 and ω -6 PUFAs, where ω -3 PUFAs exhibit beneficial effects, such as the suppression of cardiovascular events and the improvement of abnormal lipid metabolism. However, their effects on rheumatoid arthritis (RA) are unclear. Epoxy fatty acids, which are intermediate metabolites of ω -6 and ω -3 PUFAs, are formed through the action of cytochrome P and possess anti-inflammatory properties. However, they are quickly converted into their inactive forms by soluble epoxide hydrolase.

Methods: The protective effects of ω -3 and ω -6 PUFAs on arthritic symptoms with or without soluble epoxide hydrolase inhibitors (sEHIs) were investigated in a collagen-induced arthritis mouse model.

Results: The group treated with a combination of ω -3 PUFAs and sEHi showed a marked inhibition of arthritis compared to the other groups. Analysis of the faecal flora before the onset of arthritis showed an inverse correlation between the arthritis score and the relative abundance of *Lactococcus*, suggesting that changes in the intestinal flora in the preclinical stage may influence the onset of arthritis.

Conclusions: This study highlights the potential of ω -3 PUFAs combined with sEHi as the novel protective effect for RA.

KEYWORDS: Collagen-induced arthritis; gut microbiota; polyunsaturated fatty acids; rheumatoid arthritis; soluble epoxide hydrolase inhibitors

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that induces pain and swelling in the joints. Elevated levels of proinflammatory cytokines, such as Tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-17, and IL-33, contribute to synovial inflammation and joint destruction [1]. For RA, disease-modifying antirheumatic drugs are administered to achieve a treat-to-target strategy [2]. In recent years, biological disease-modifying antirheumatic drugs, which target proinflammatory cytokines, have been confirmed as effective options for RA management. However, not all patients benefit from these drugs, partly due to the side effects of immunosuppression and high costs. Therefore, there is a need for cost effective drugs with fewer adverse effects.

For a considerable period, specific dietary contents and eating habits have been proposed as nonpharmacological strategies to manage RA. Particularly, the balance between ω -6 and ω -3 polyunsaturated fatty acids (PUFAs) has been argued [3]. The ratio of ω -6 to ω -3 PUFA intake is in favour of ω -6 [4], and a higher intake of ω -3 PUFA supplementation has been shown to inhibit arthritis in mouse models through increasing the expression of FoxP3 and the differentiation of Treg [5]. However, in humans, the antirheumatic effects of ω -3 PUFA have been investigated in several clinical trials, most of which have revealed relatively modest results [6].

PUFAs are metabolized via multiple enzymatic pathways, including cyclooxygenase, lipoxygenase, and cytochrome P

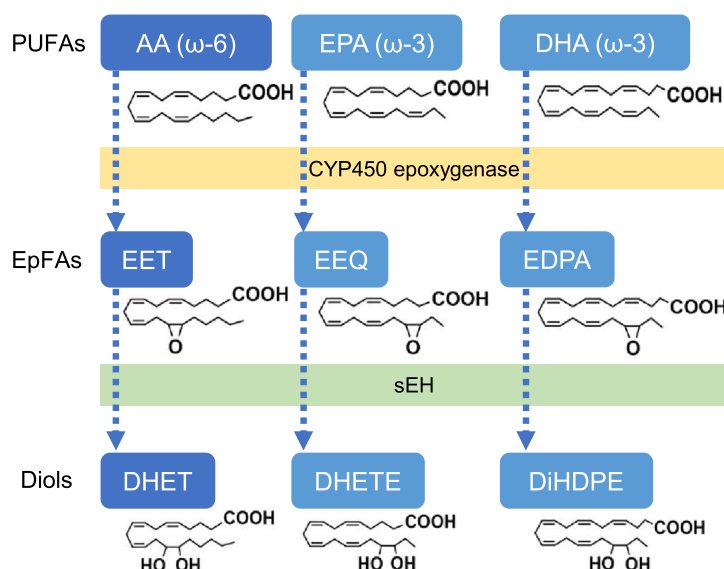


Figure 1. Overview of the CYP epoxygenase-mediated metabolism of ω -6 and ω -3 PUFAs. Each PUFA (AA, EPA, and DHA) can be converted to EpFAs (EET, EEQ, and EDPA) by CYP epoxygenases. EpFAs are unstable and quickly metabolized to less active diols by sEH. Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DHET, dihydroxyeicosatrienoic acid; DHETE, dihydroxyeicosatetraenoic acid; DiHDPE, dihydroxydocosapentaenoic acid; EDPA, epoxydocosapentaenoic acids; EEQ, epoxy eicosatetraenoic; EPA, eicosapentaenoic acid.

(CYP) pathways, into a variety of metabolites with different physiological activities [7]. Epoxy fatty acids (EpFAs) are intermediate metabolites of PUFAs that are epoxygenated by CYP450 epoxygenase (Figure 1). There are two main EpFA families, namely, ω -3 and ω -6. Arachidonic acid, a ω -6 PUFA, forms epoxyeicosatrienoic acid (EET) and has frequently been reported to have anti-inflammatory, vasodilatory, antiallergic, and antioxidative effects [8]. Similar to EET, eicosapentaenoic acid and docosahexaenoic acid are well-known ω -3 PUFAs that form epoxy eicosatetraenoic acid and epoxydocosapentaenoic acids, respectively [8]. The anti-inflammatory effects of EETs have been described in multiple animal models, namely, diabetes, cardiovascular disease, stroke, traumatic brain injury, Parkinson's disease, epilepsy, cognitive impairment, dementia, depression, and neuropathic pain [9–11]. The ω -3 EpFAs were initially considered to have a similar or lower therapeutic effect than EET. However, reports have confirmed that ω -3 to ω -6 EpFAs effects are different [12, 13].

EpFAs are quickly converted into their inactive metabolites by soluble epoxide hydrolase (sEH) [14]. Elevated sEH levels have been reported in the liver and brains of patients with depression [15, 16], Parkinson's disease [17], and Alzheimer's disease [18, 19]. Elevated levels have also been reported in the synovium and fibroblast-like synoviocytes in patients with RA [20]. Accordingly, sEH has been explored as a therapeutic target, and sEH inhibitors (sEHi) have been reported to suppress arthritis *in vivo* [21, 22].

Oral lipid intake also affects the intestinal environment. Intestinal immunity plays an important role in the pathogenesis and inflammation of RA [23]. Omega-3 PUFAs affect the gut microbiota in three main ways: (1) modulating the type and abundance of gut microbes; (2) altering the levels of proinflammatory mediators, such as endotoxins (lipopolysaccharides) and IL-17; and (3) regulating the levels of short-chain fatty acids (SCFAs) or SCFA salts [24]. In adults, changes in the gut microbiota have been observed after ω -3 PUFA supplementation [25].

Based on this background, it was hypothesized that PUFAs and sEHi could alter the intestinal microbiota. Considering this information, a combination of ω -3 rich PUFAs and sEHi might offer greater benefits against various diseases. In this study, the effects of a combination therapy on inflammatory arthritis were investigated.

Materials and methods

Animals and animal care

Cohoused DBA/1J male mice (SLC Inc., Shizuoka, Japan), which were not littermates, weighing 25–30 g, were used in this study. The mice were raised under specific pathogen-free conditions and kept in cages (four per cage) in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) with a 12:12 h light dark cycle. They were provided water and food *ad libitum*. All procedures were performed in accordance with the standards for the care of laboratory animals outlined in the guidelines 'Regulations on Animal Experimentation at Kyoto University' (revised 2020). The study received ethical approval from the university's internal ethics committee (approval number Medkyo 24083).

Treatments

Soluble epoxide hydrolase inhibitor 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl] urea (TPPU, #HY-101294; MedChemExpress, Monmouth Junction, NJ, USA) was utilized. This was synthesized, purified, and chemically characterized in house, as described previously [17]. The TPPU was dissolved in polyethylene glycol (PEG) 400, and the mice were treated with either TPPU (10 mg/kg by gavage) or a vehicle (the same volume of PEG 400 in saline) daily. Three diet formulations were prepared as described in Table 1 (Research Diets Inc, New Brunswick, NJ, USA). All formulations used the purified American Institute of

Table 1. Concentrations of fatty acids in the experimental diet.

Diet	Control g/%	kcal%	ω -6-rich g/%	kcal%	ω -3-rich g/%	kcal%
Protein	20	20	20	20	20	20
Carbohydrate	64	64	64	64	64	64
Fat	7	16	7	16	7	16
Total		100		100		100
kcal/gm	4		4		4	
Ingredients	g	kcal	g	kcal	g	kcal
Casein	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Corn starch	397.486	1590	397.486	1590	397.486	1590
Maltodextrin 10	132	528	132	528	132	528
Sucrose	100	400	100	400	100	400
Cellulose, BW200	50	0	50	0	50	0
Corn oil	0	0	20.8	187	6.5	59
Nonhydrogenated coconut oil	0	0	49.2	443	49.2	443
Menhaden oil (200 ppm <i>t</i> -BHQ)	0	0	0	0	14.3	129
Soybean oil	70	630	0	0	0	0
<i>t</i> -BHQ	0.014	0	0.014	0	0.011	0
Mineral mix S10037	35	0	35	0	35	0
Vitamin mix V10037	10	40	10	40	10	40
Choline bitartrate	2.5	0	2.5	0	2.5	0
Cholesterol	0.084	0	0.084	0	0	0
Total	1000.084	4000	1000.084	4000	1000.047	4000
Cholesterol (mg/kg)	124		124		124	
<i>t</i> -BHQ (g/kg)	0.014		0.014		0.0139	

Casein and menhaden oils are estimated to contain 20 and 588 mg/100 g cholesterol, respectively. Abbreviation: *t*-BHQ, *t*-Butylhydroquinone.

Nutrition-93G diet (70 g/kg fat) as the base to provide optimal nutrition to the experimental mice. For the ω -6- and ω -3-rich diets, the amounts of corn, coconut, and fish (menhaden) oils were adjusted in accordance with a previous study [26], such that the ω -6-rich food has a ratio of ω -6/ ω -3 of 60:1, which is the ratio referred to in western diets. The ω -3-rich diet had a ratio of ω -6/ ω -3 of 1:1, which is the ratio recommended for humans. Control diets were American Institute of Nutrition-93G with matched amounts of cholesterol and *t*-butylhydroquinone added to the ω -6- or ω -3-rich diet. In Study 1, special diets and additive water were provided after the first immunization; in Study 2, the treatments started before the first immunization. Noncollagen-induced arthritis mice were provided special diets and additive water for 14 days from 7 weeks old.

Collagen-induced arthritis in DBA/1J mice

Collagen-induced arthritis (CIA) was induced in mice according to a previously described protocol [27]. Male DBA/1J mice (8–11 weeks) were immunized intradermally with 100 μ g of chicken sternal hyaline collagen type 2 (CII, #20011; Chondrex Inc., Woodinville, WA, USA) that was dissolved in 100 μ l acetic acid (0.05 mol/l) and mixed with an equal volume of complete Freund's adjuvant (CFA, #7023; Chondrex Inc.) at a location 1.5 cm from the base of the tail. After days, the animals were boosted with 100 μ g of CII emulsified in incomplete Freund's adjuvant (#7002; Chondrex Inc.). The mice were monitored approximately every third day for signs of arthritis. Arthritis scores were assigned based on erythema or swelling in each paw on a scale of 0–1.5, with a maximum score of 6 per mouse. Articular oedema of the footpad was assessed by measuring the transverse diameter using a digital calliper.

Clinical score

Mice were inspected for the development of CIA, and the inflammation of all four paws per mouse was graded between 0 and 1.5 (0: paws with no swelling; 0.5: paws with mild swelling of wrist or ankle joints; 1: severe swelling of the entire paw; and 0.1 point was added for each swollen finger). Each paw was graded, and the four scores were added. Scoring was performed by two blinded researchers.

Histological analysis

Mice in Study 1 were sacrificed on Day 37 through cervical dislocation, and the left ankle joints were collected for histological analysis. The left leg was amputated with scissors about 1 cm proximal to the ankle joint. Ankle joints were fixed in 10% neutral-buffered formalin for 2 days before decalcification in an NaOH/ethylenediaminetetraacetic acid solution (pH 7.4) and processed for paraffin embedding. Longitudinal tissue sections (5 μ m) were prepared and stained with haematoxylin and eosin (H&E). The H&E-stained femorotibial joint sections were examined using light microscopy, and the degree of vascular proliferation and inflammatory infiltration was determined and scored. The degree of synovial inflammation was classified on a scale of 0 to 3 [0, no inflammation (absence of inflammatory cells); 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation]. The degree of bone erosion was classified on a scale of 0 to 3 (0, intact bone surface; 1, superficial bone erosion; 2, enhanced focal subchondral bone erosion; 3, loss of bone architecture). These scores were determined in reference to a previous study [28]. Scoring was performed by two researchers, blinded, and the mean score was adopted.

Another section of the ankle joint was stained with tartrate-resistant acid phosphatase (TRAP; LLC, #387A; Sigma-Aldrich Co., St. Louis, MI, USA), which was an enzyme

that is particularly abundant in osteoclasts, the cells responsible for bone resorption. Osteoclasts in the tibial and talar cartilage areas were counted using the Hybrid Cell Count Module (Keyence Co., Osaka, Japan). The TRAP-positive cells were defined as cells with more than three nuclei and 100 μm immunity per cell.

Enzyme-linked immunosorbent assay and cytokine analysis

Plasma was collected from mice 35 days after the first immunization with CII/CFA. It was diluted 1:100,000 and analysed using a mouse anti-type 2 collagen IgG enzyme-linked immunosorbent assay kit (#2036T; Chondrex Inc), according to the manufacturer's instructions. Plasma cytokine levels were determined using mouse IL-17A Enzyme-Linked Immuno-Sorbent Assay kit (# KE10020; Proteintech Inc, Rosemont, IL, USA) and U-PLEX Biomarker Group 1 (mouse) multiplex assay (LLC, #K15355K; Meso Scale Diagnostics, Rockville, MD, USA), according to the manufacturer's instructions.

Bacterial DNA extraction and 16S ribosomal RNA gene sequencing

The faeces of CIA mice were collected 14 days before the first immunization and on Day 25 in Study 1. The faeces of non-CIA mice were collected 7 weeks old and after 14 days with special feeding. The faeces were dried using a VD-250R freeze dryer (TAITEC Co., Aichi, Japan) and homogenized for 2 min using a multi-bead shaker (Yasui Kikai Co., Osaka, Japan). Samples were mixed with lysis solution F (#315-06281; Nippon Jean Co., Ltd., Tokyo, Japan) and incubated at 65°C for 10 min. After centrifugation at 12,000 $\times g$ for 2 min, the supernatant was collected. Bacterial genomic DNA was extracted using a Lab-Aid824s DNA extraction kit (#607106; ZEESAN Biotech Co., Ltd., Fujian, China), according to the manufacturer's protocol. A 16S ribosomal RNA genomic library was constructed using the Illumina protocol. The extracted DNA samples were used as DNA templates for polymerase chain reaction in a reaction mixture containing template DNA, KOD FX Neo (#KFX-201; TOYOBO Co., LTD., Osaka, Japan) and forward

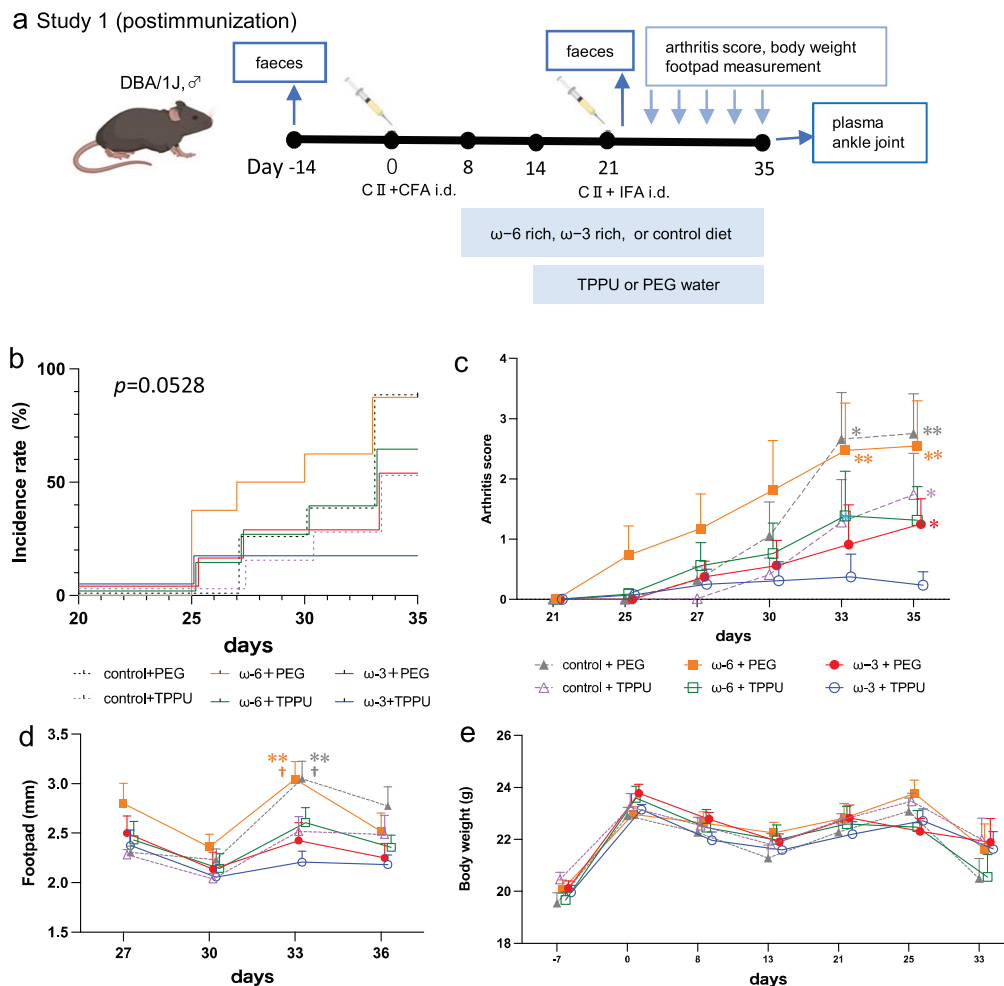


Figure 2. Effect of PUFA and TPPU combination treatment on CIA. (a) Experimental design for Study 1. At 11 weeks of age, male DBA/1J mice ($n=8/\text{gp}$, 6 gps) were immunized on Day 0 via the i.d. route, with CII and CFA. Cholesterol-added diet (control, ω -6- or ω -3-rich) was started on Day 8, and additive water (TPPU or PEG water) was started on Day 14. Second immunization was done on Day 21 with CII and incomplete Freund's adjuvant. Mice were sacrificed on Day 37. Faeces were collected 14 days before the first immunization and on Day 25. (b) Incidence rate of CIA. Log-rank test. (c) Arthritis score. (d) Footpad thickness. (e) Body weight. Values represent mean $\pm$ SEM per group. * $P < .05$ vs. ω -3 + TPPU group; ** $P < .01$ vs. ω -3 + TPPU group; † $P < .05$ vs. ω -3 + PEG group; Kruskal–Wallis analysis followed by the *post hoc* Wilcoxon test. i.d.: intradermic injection.

(5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG-3') and reverse (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNN-GACTACHVGGGTATCTAATCC-3') primers for the 16S ribosomal RNA V3-V4 gene region. Initial denaturation was performed at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 30 s. The final elongation step was performed at 68°C for 7 min. The amplicons were purified with AMPure XP beads (#A63880; Beckman Coulter, Brea, CA, USA) and attached to dual indices using index Polymerase Chain Reaction (PCR) with ExTaq HS (#RR006A; TAKARA BIO Inc., Shiga, Japan) and forward (5'-AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC-3') and reverse (5'-CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3') primers. The libraries were purified using AMPure XP beads (Beckman Coulter). The purified libraries were diluted to 4 nM in Tris-HCl buffer and pooled. The libraries were sequenced on a MiSeq (Illumina Co., San Diego, CA, USA) with 300-bp paired-end reads. The

sequenced PCR amplicons from sterile water or DNA extracts from empty samples were used as negative controls.

Microbiome analysis

Extract reads that matched the primer sequence exactly at the start were extracted using the FASTX-Toolkit `fastx_barcode_splitter` tool (ver. 0.0.14). The FASTQ files were analysed using the QIIME2 pipeline (QIIME2 version 2022.8). After converting the files to the QIIME Zipped Artifacts format, the sequenced data were demultiplexed and summarized using QIIME2 paired-end-demux. The sequences were trimmed and denoised using the `dada2` plugin of QIIME2. Taxonomic assignment was performed using a Naïve Bayes fitted classifier trained on the SILVA_138 reference database (Silva 138 SSURF NR99) with a feature classifier plugin for QIIME2. The phylogenetic tree for diversity analysis was reconstructed using the QIIME2 `align-to-tree-mafft-fasttree`. Diversity analysis was performed using QIIME2 core-metric `s-phylogenetic` analysis. The relative abundance of each taxon was calculated

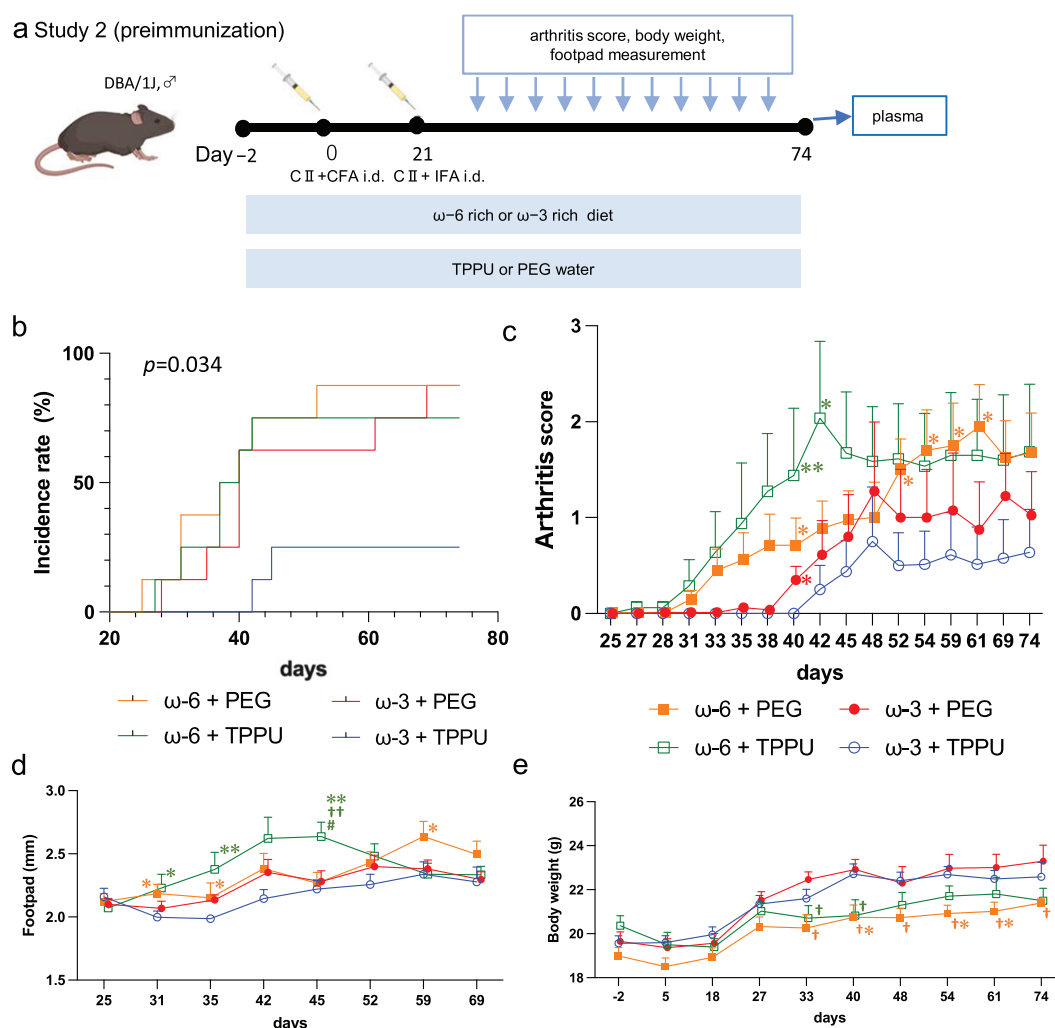


Figure 3. ω-3 PUFA and TPPU combination treatment on CIA. (a) Experimental design for Study 2. At 8 weeks of age, DBA/1J mice ($n=8/\text{gp}$, 4 gps) were induced for CIA. Cholesterol-added diet (ω-6- or ω-3-rich) and additive water (TPPU or PEG water) started 2 days before the first immunization. Mice were sacrificed on Day 75. (b) Incidence rate of CIA. Log-rank test. (c) Arthritis score. (d) Footpad thickness. (e) Body weight. Values represent mean $\pm$ SEM per group. * $P < .05$ vs. ω-3 + TPPU group; ** $P < .01$ vs. ω-3 + TPPU group; † $P < .05$ vs. ω-3 + PEG group; ‡ $P < .01$ vs. ω-3 + PEG group; # $P < .01$ ω-6 + TPPU vs. ω-6 + PEG, Kruskal-Wallis analysis followed by the *post hoc* Wilcoxon test.

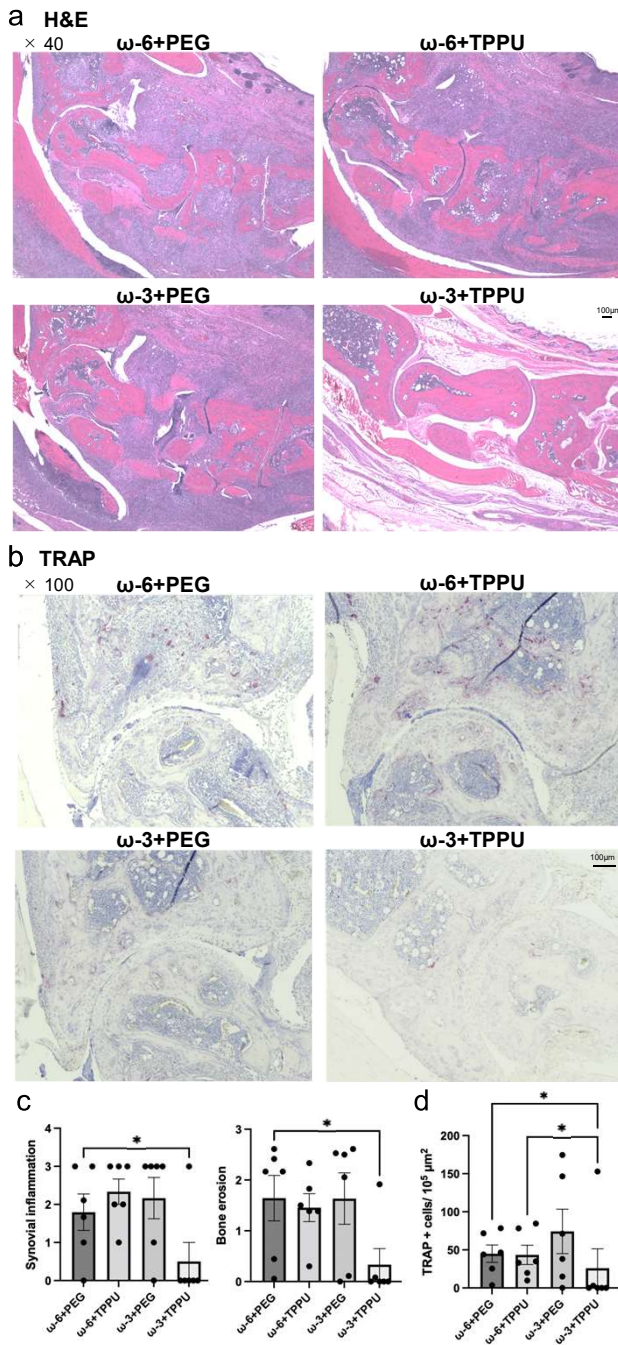


Figure 4. Pathological results of PUFA and TPPU combination therapy. (a) Representative histology of the ankle joint on Day 35 from groups with PUFA-rich diet in Study 1. (b) Representative TRAP staining histology. (c) Synovial inflammation and bone erosion score ($n=6$ /gp, 4 gps). (d) The number of TRAP-positive and multinuclei ($n>2$) cells per area. Values represent the mean $\pm$ SEM of six mice per group. * $P<.05$, Kruskal–Wallis analysis followed by the *post hoc* Wilcoxon test.

using the taxa collapse QIIME2 plugin. Pathway analysis was performed using PICRUSt2, and the description of each pathway was verified using MetaCyc (<https://metacyc.org>).

Statistical analysis

Statistical analyses were performed using the Prism 9.0 and JMP software. Differences between the mean values were analysed using two-way analysis of variance (ANOVA) or

the Kruskal–Wallis test followed by the Wilcoxon test. P values $<.05$ were considered significant. Data are presented as mean $\pm$ SEM. Bacterial taxa were compared at the genus level using the linear discriminant analysis effect size method [29] (<https://huttenhower.sph.harvard.edu/galaxy/root>). Differences in β -diversities between the two groups were analysed using permutational multivariate ANOVA (PERMANOVA).

Results

Combined effects of PUFAs and sEHi on CIA

The experimental design for Study 1 is shown in Figure 2a. The cholesterol-added diet (control, ω -6 PUFA-rich, or ω -3-PUFA-rich) started 8 days after the first immunization, and additive water of TPPU or PEG (vehicle control) started from Day 14. In Study 1, mice in the ω -3 PUFA + TPPU combination group showed a lower incidence of arthritis (Figure 2b). Mean arthritis score and swelling of the feet for the ω -3 PUFA + TPPU combination group was strongly suppressed compared to that for the other groups (Figure 2c and d). Moreover, the TPPU showed protective effectiveness in each diet group. The ω -6 PUFAs and control diet groups showed a similar clinical course. No significant difference in body weight was noted between the groups ($P>.05$; Figure 2e).

To examine the preventive effect of ω -3 PUFAs and TPPU on CIA, ‘Study 2’ was performed in which the cholesterol-added diet (ω -6 PUFA-rich or ω -3 PUFA-rich) and additive water (TPPU or PEG) started 2 days before the first immunization (Figure 3a). No control diet groups were set in this study because the control diet and ω -6 PUFA-rich diet groups showed similar results to those in Study 1. The ω -3 PUFA + TPPU combination group suppressed the onset of arthritis and remained suppressed during the 74 days of observation (Figure 3b). Mean arthritis scores and swelling of the feet were suppressed for the ω -3 PUFA + TPPU combination group (Figure 3c and d). After Day 33, body weight was lower in the ω -6 PUFA groups (Figure 3e), possibly due to chronic inflammation.

Histopathological articular changes in CIA

Histopathological sections of the ankle joints on Day 35 in Study 1 showed intense synovial and inflammatory cell proliferation and infiltration, respectively, and bone erosion in the arthritis-prone mice. However, these changes were not observed in most H&E-stained sections from mice in the ω -3 PUFA + TPPU group (Figure 4a). Fewer cells were positive for TRAP staining in the ω -3 PUFA + TPPU group (Figure 4b). Pathological scores were significantly decreased in the ω -3 PUFA + TPPU group (Figure 4c). The number of TRAP-positive cells was also lower in the ω -3 PUFA + TPPU group (Figure 4d).

Levels of anti-type 2 collagen IgG antibodies and proinflammatory cytokines in plasma

Plasma titres of the anti-CII IgG antibody were lowest in the ω -6 PUFA + TPPU group on Day 35 in Study 1 (Figure 5a). No differences in the plasma IgG titre were observed between the groups on Day 75 in Study 2 (Figure 5b). Additionally, plasma levels of IL-6, TNF- α , and interferon (IFN)- γ were adequate in the ω -3 PUFA + TPPU group (Figure 5c).

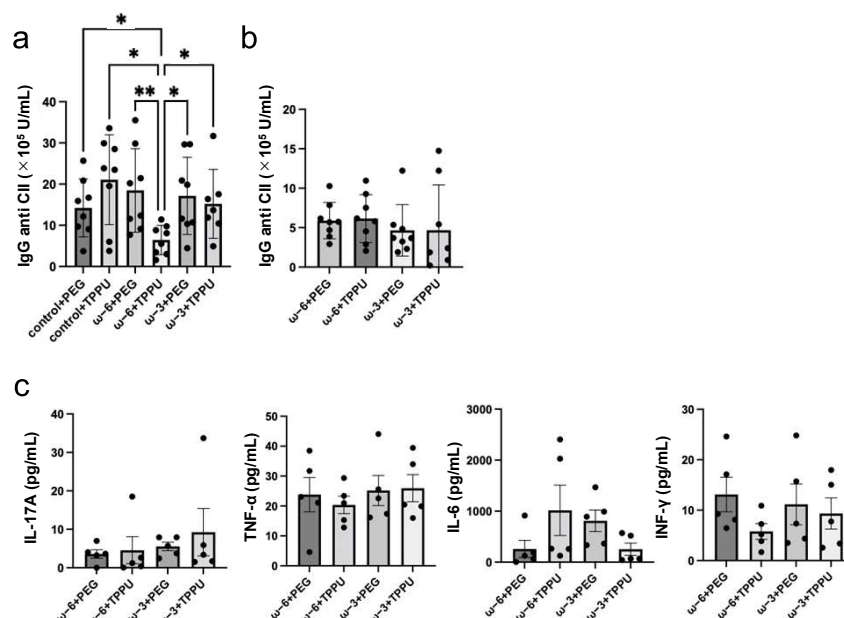


Figure 5. Assessment of systemic immune response using plasma. Anti-IgG titres in plasma on Day 35 in Study 1 (a) and on Day 75 in Study 2 (b). Values represent mean $\pm$ SEM of eight mice per group. * $P < .05$, ** $P < .1$, Kruskal–Wallis analysis followed by the *post hoc* Wilcoxon test. (c) Plasma levels of proinflammatory cytokines were measured on Day 35 in Study 1. Values represent the mean $\pm$ SEM for five mice per group.

Gut microbiota between the ω -6 and ω -3 PUFA + TPPU groups before the onset of CIA

The faeces of mice were collected 2 weeks before the first injection (preimmunization) and on Day 25 (after the second injection and before the occurrence of arthritis) in Study 1. The composition of intestinal microbiota at the family level was different in mice before and after CIA immunization (Figure 6a). Alpha diversity analysis (species richness, Chao1, Shannon entropy, and Faith's phylogenetic diversity) tended to be lower in the ω -3 PUFA + TPPU group than in the other groups, but not significantly (Figure 6b). Regarding β -diversity, unweighted unifrac showed a significant difference between the ω -6 PUFA + PEG and ω -3 PUFA + TPPU groups ($P < .05$; Figure 6c). Comparative analysis of bacteria at the species level revealed characteristic strains in each group (Figure 7a). Some strains overlapped between groups, but *Lactococcus* was specific to the ω -3 PUFA + TPPU group. The relationship between the relative abundance of strains and arthritis score was examined. A negative correlation was observed for *Lactococcus*, and a positive correlation was evident for *Ruminococcaceae* and *Anaerotruncus* (Figure 7b). On the other hand, in the non-CIA mice, the number and types of bacterial species that differed between groups were also different from those of CIA mice (Supplement Figure 1a and b). *Lactococcus* was prominent in the CIA situation. The proportion of *Lactococcus* tended to decrease in CIA mice compared to non-CIA mice, but only in the ω -3 PUFA + TPPU group, the proportion increased in the CIA group (Supplement Figure 2a).

Metabolic pathway analysis of mouse faeces

The metabolic pathway analysis of the faeces revealed differences in many pathways (Table 2). Pathways involved in vitamin K1 (phyloquinone) and vitamin K2 (menaquinone) biosynthesis and SCFA production were identified. In this

analysis, the contribution of dietary factors was greater than that of TPPU. All pathways classified as vitamin K biosynthesis ('Menaquinol Biosynthesis', 'Demethylmenaquinol Biosynthesis', 'Phylloquinol Biosynthesis', and '1,4-Dihydroxy-2-Naphthoate Biosynthesis') and 'Fermentation to Short-Chain Fatty Acids' on the MetaCYC (<https://metacyc.org>) were identified, and heat maps were created based on the normalized values for each sample (Figure 8a and b). The vitamin K2 biosynthesis pathway was highly enhanced in the ω -3 PUFA + TPPU group, whereas the pathways for SCFA production were enhanced in the ω -3 PUFA + TPPU and ω -6 PUFA + PEG groups. All the vitamin K biosynthesis pathways, which showed significant difference ($P < .001$) in the two-way ANOVA, were enhanced in the group order of ω -3 PUFA + TPPU > ω -3 PUFA + PEG > ω -6 PUFA + TPPU > ω -6 PUFA + PEG. *Post hoc* Wilcoxon testing shows significant differences among ω -3 PUFA + TPPU vs ω -6 PUFA + PEG ($P < .01$), ω -3 PUFA + TPPU vs ω -6 PUFA + TPPU ($P < .01$), and ω -3 PUFA + PEG vs ω -6 PUFA + PEG ($P < .05$).

Discussion

sEH is a widely distributed bifunctional enzyme that contains C-terminal epoxide hydrolase and N-terminal phosphatase domains [30]. EpFAs are hydrolysed to corresponding diols and contributes to various inflammatory conditions. Therefore, sEH inhibition is thought to exert anti-inflammatory effects via the accumulation of EpFAs such as EET, EEQ, and EDPA. TPPU is a novel sEHi synthesized in 2012, because of its better absorption rate and stability from the intestinal tract. It has been used in basic research in various cardiovascular and cerebrovascular diseases, for features to cross the cerebrovascular barrier [31].

In the present study, the combination of ω -3 PUFAs and TPPU exhibited a strong inhibitory effect on the development of arthritis. Contrary to the clinical score, the

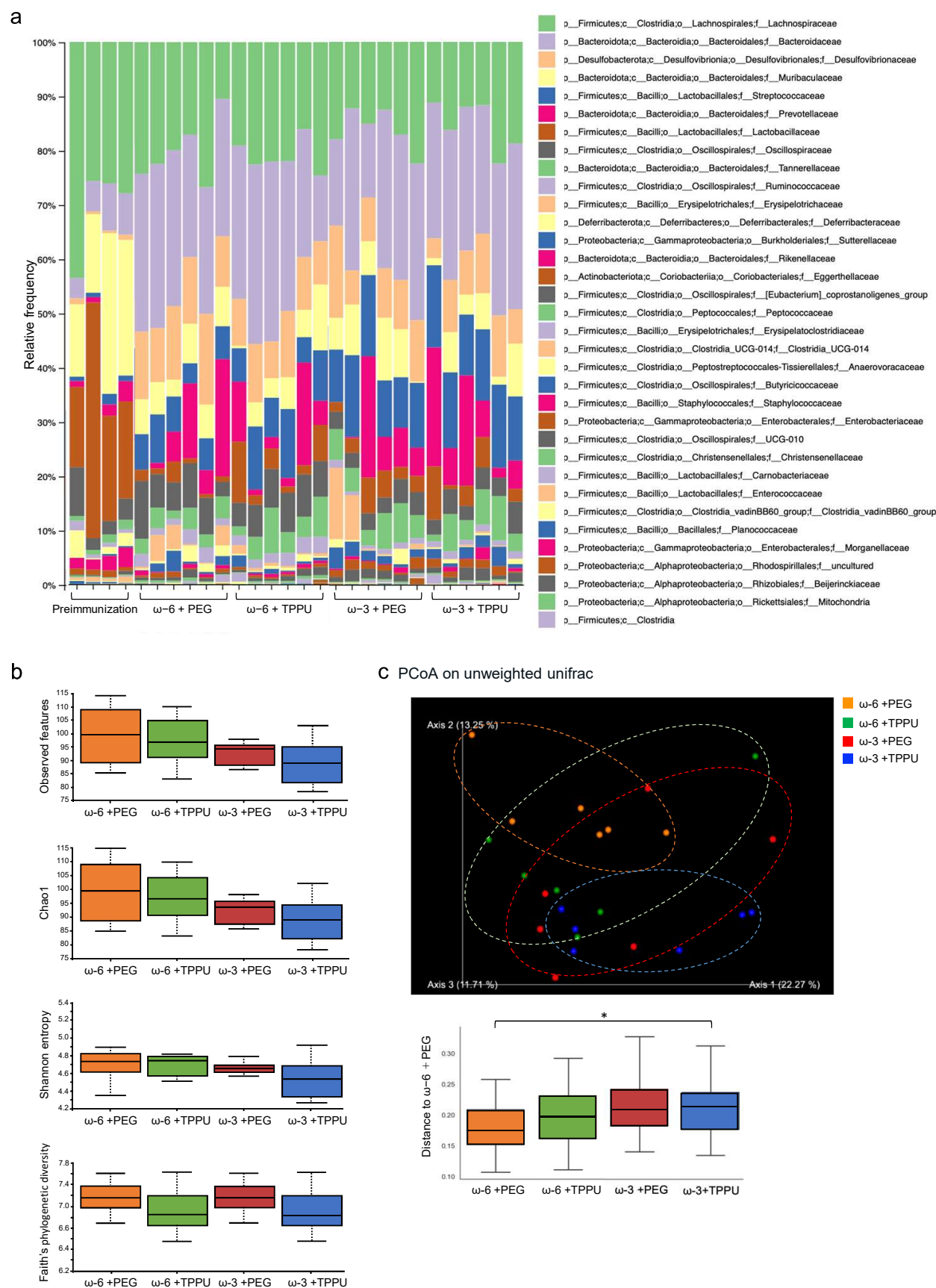


Figure 6. Changes in the composition of intestinal bacteria. (a) Relative frequency of the family-level bacterial composition in faeces from each sample. Preimmunization samples were collected 2 weeks before the first immunization and others were collected on the 25th day of Study 1. (b) Analysis of α -diversity (observed features, Chao1, Shannon entropy, and Faith's phylogenetic diversity) in each group. (c) Analysis of β -diversity. Values represent the mean $\pm$ SEM for six mice per group. * $P < .05$ PERMANOVA. Taxon levels are described as p: Phylum; c: Class; o: Order; and f: Family.

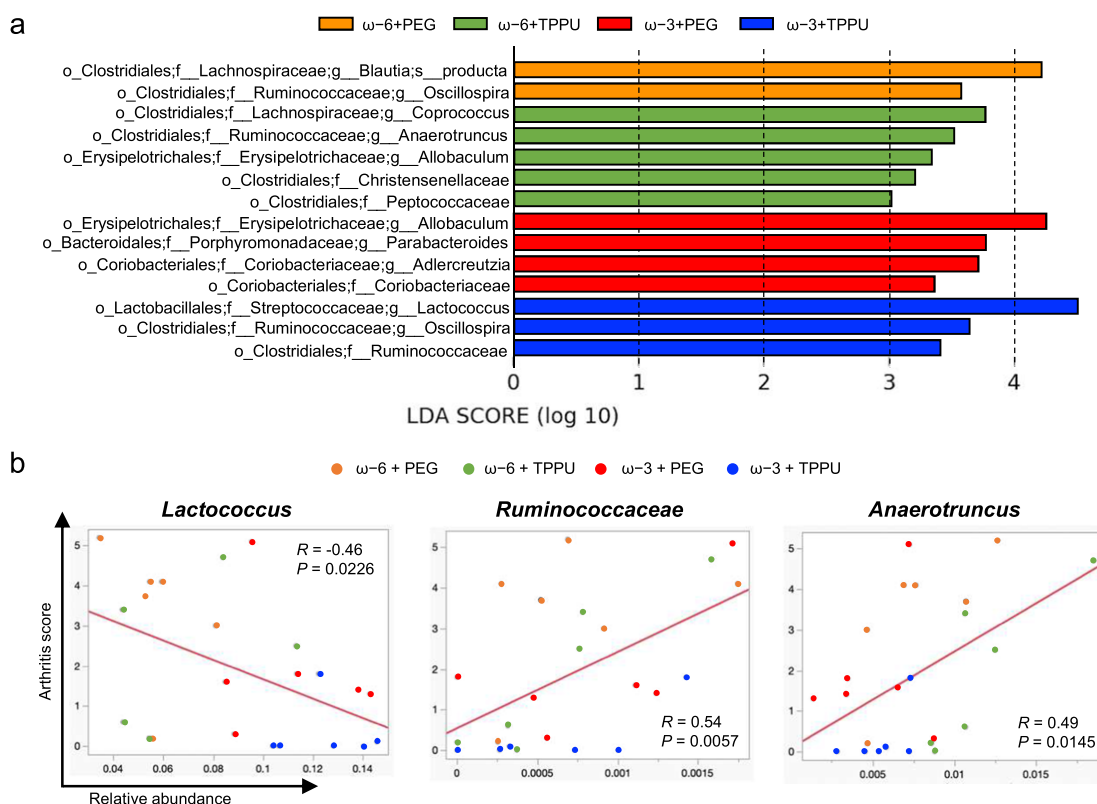


Figure 7. Differences in the relative abundance of gut microbiota. (a) LfSE analysis. Histogram of liner discriminant analysis (LDA) representing significant differences in the abundance of gut microbiota among the four groups (ω -6 + PEG, ω -6 + TPPU, ω -3 + PEG, and ω -3 + TPPU). (b) Correlation between the relative abundance of bacteria and the arthritis score. $n = 6$ mice per group. R : correlation coefficient. Taxon levels are described as o: Order; f: Family; g: Genus; and s: Species. LfSE: Linear discriminant analysis Effect Size.

ω -3 PUFA + TPPU group had a high IgG titre and adequate plasma levels of proinflammatory cytokines. These results suggest that the mechanism by which arthritis is inhibited by the ω -3 PUFA and TPPU treatments is not through the suppression of IgG production. We have examined whether the same tendency would be observed in anti-type 2 collagen antibody induced arthritis (CAIA), but no significant difference was observed (data not shown). It is well-known that CAIA is suitable for exploring the inflammatory phase of arthritis, while CIA for understanding autoimmune initiation and progression. This difference might be one of the reasons to account for the effect of ω -3 EpFAs. First, we hypothesized that differences in IgG pathogenicity had occurred. Antibiotic administration can suppress disease progression in CIA and systemic lupus erythematosus model mice and that changes in the gut microbiota may increase sialylation of IgG-Fc [32, 33]. IgG that has sialic acid added to it has a lower proinflammatory effect compared to IgG without sialic acid [34]. Second, regulatory T cell activity exerts the potential suppression of abnormal synovial cell. sEHi already reported increases regulatory T cell pathway in an arthritis model [21]. Third, local synovial membrane proliferation and bone destruction are inhibited in many courses. sEHi inhibits the activation of nucleotide-binding domain leucine-rich repeat-containing receptor, pyrin domain-containing-3 (NLRP3) inflammasome in macrophages, which plays a crucial role in CIA not in CAIA [35, 36]. In the CAIA model, it has been reported that even when apoptosis-associated

speck-like protein containing a caspase recruit domain, a component of the NLRP3 inflammasome, is knocked out, there is no significant effect on the severity of arthritis [37]. In addition, sEHi was reported to inhibit osteoclast differentiation, protect chondrocytes from cytokine-induced apoptosis *in vivo* [38], and suppress the abnormal activation of fibroblast-like synoviocytes in patients with RA [20]. Omega-3 PUFAs inhibit osteoclastogenesis by decreasing the osteoprotegerin/RANKL signalling pathway and protecting cartilage from oxidative stress and apoptosis [39]. Accordingly, ω -3-rich PUFAs with TPPU therapy can get benefit from both aspects.

The ω -6 + TPPU group showed the highest suppression of IgG production in Study 1 but not the lowest arthritis score. One reason is that the arthritis score in Study 1 may have entered the peak-out phase earlier than the other groups. The ω -6 PUFA and sEHi group also tended to peak earlier than the others in Study 2. Second, sEHi under ω -6 PUFA-rich conditions may suppress IgG production but enhance local inflammation. EETs (ω -6 EpFA) were reported to inhibit antibody production of B lymphocytes in mice [40]. On the other hand, sEHi treatment also affected lipoxygenase- and cyclooxygenase-mediated metabolite concentrations. For example, inflammatory lipid mediators such as leukotriene B₄, prostaglandin E₂, and prostaglandin F₂ α , which are metabolites of arachidonic acid, and proinflammatory lipid mediators such as E-series resolvins, which are metabolites of eicosapentaenoic acid, are increased by sEHi [41]. This is

Table 2. List of the pathways with significant differences among groups.

Pathway	Description	P	Main effect
PWY-5837	1,4-Dihydroxy-2-naphthoate biosynthesis I ^a	<.0001	ω-3 PUFA
PWY-5863	Superpathway of phylloquinol biosynthesis ^a	<.0001	ω-3 PUFA
PWY-5861	Superpathway of demethylmenaquinol-8 biosynthesis ^a	<.0001	ω-3 PUFA
PWY-5897	Superpathway of menaquinol-11 biosynthesis ^a	<.0001	ω-3 PUFA
PWY-5898	Superpathway of menaquinol-12 biosynthesis ^a	<.0001	ω-3 PUFA
PWY-5899	Superpathway of menaquinol-13 biosynthesis ^a	<.0001	ω-3 PUFA
PWY-5840	Superpathway of menaquinol-7 biosynthesis ^a	<.0001	ω-3 PUFA
PWY0-1061	Superpathway of L-alanine biosynthesis	<.0001	ω-3 PUFA
PWY-5838	Superpathway of menaquinol-8 biosynthesis I ^a	<.0001	ω-3 PUFA
REDCITCYC	Reductive TCA cycle	<.0001	ω-6 PUFA
PWY-5676	Acetyl-CoA fermentation to butanoate ^b	<.0001	ω-6 PUFA
P163-PWY	L-Lysine fermentation to acetate and butanoate ^b	<.0001	ω-6 PUFA
PWY-2941	L-Lysine biosynthesis II	.0002	ω-3 PUFA, TPPU
P461-PWY	Hexitol fermentation to lactate, formate, ethanol, and acetate ^b	.0002	ω-3 PUFA
PWY-6588	Pyruvate fermentation to acetone	.0002	ω-6 PUFA
TEICHOICACID-PWY	Teichoic acid (poly-glycerol) biosynthesis	.0004	ω-3 PUFA
PWY-5910	Superpathway of geranylgeranyl diphosphate biosynthesis I (via mevalonate)	.0005	ω-3 PUFA, TPPU
PWY-922	Coenzyme M biosynthesis I	.0006	ω-3 PUFA, TPPU
FAO-PWY	Fatty acid and beta-oxidation I	.0007	ω-6 PUFA
P122-PWY	Heterolactic fermentation ^b	.0008	ω-3 PUFA, TPPU
P261-PWY	Coenzyme M biosynthesis I	.0008	ω-3 PUFA
PWY-7234	Inosine-5'-phosphate biosynthesis III	.001	ω-3 PUFA
PWY-6590	Superpathway of <i>Clostridium acetobutylicum</i> acidogenic fermentation ^b	.0012	ω-6 PUFA
CENTFERM-PWY	Pyruvate fermentation to butanoate ^b	.0012	ω-6 PUFA
PWY-7013	S-1,2-Propanediol degradation ^b	.0015	ω-6 PUFA
PWY-6471	Peptidoglycan biosynthesis IV (<i>Enterococcus faecium</i>) biosynthesis	.0023	ω-3 PUFA, TPPU
PWY-5505	L-Glutamate and L-glutamine biosynthesis	.0024	ω-6 PUFA
PWY-5845	Superpathway of menaquinol-9 biosynthesis ^a	.0024	ω-3 PUFA
PWY-5850	Superpathway of menaquinol-6 biosynthesis ^a	.0024	ω-3 PUFA
PWY-5896	Superpathway of menaquinol-10 biosynthesis ^a	.0024	ω-3 PUFA
PWY0-781	Aspartate superpathway	.0024	ω-3 PUFA
PWY-5177	Glutaryl-CoA degradation	.0028	ω-6 PUFA
PWY-5860	Superpathway of demethylmenaquinol-6 biosynthesis I ^a	.0030	ω-3 PUFA
PWY-5862	Superpathway of demethylmenaquinone-9 biosynthesis ^a	.0030	ω-3 PUFA
HOMOSER-METSYN-PWY	L-Methionine biosynthesis I	.0062	ω-3 PUFA
P124-PWY	Bifidobacterium shunt ^b	.0065	ω-3 PUFA, TPPU
PENTOSE-P-PWY	Pentose phosphate pathway	.0070	ω-3 PUFA
MET-SAM-PWY	Superpathway of S-adenosyl-L-methionine biosynthesis	.0072	ω-3 PUFA
PWY-5189	Tetrapyrrole biosynthesis II (from glycine)	.0078	ω-6 PUFA
PWY-7210	Pyrimidine deoxyribonucleotide biosynthesis from CTP	.0092	ω-6 PUFA
PWY-7198	Pyrimidine deoxyribonucleotides <i>de novo</i> biosynthesis IV	.0098	ω-6 PUFA

Pathway analysis was performed using PICRUSt2, and the description and chiral class of each pathway were referenced in MetaCyc. Only pathways that showed significant differences ($P < .01$, two-way ANOVA) are shown. TCA; tricarboxylic acid. Main effect showed significant differences at $P < .05$.

^a Pathways included in the group of vitamin biosynthesis.

^b Pathways included in the group of fermentation to SCFAs.

another reason why administering sEHi and ω-3 PUFAs at the same time is so effective.

The relationship between gut microbiota and the pathogenesis of RA has been reported in mouse models [42, 43]. The gut microbiota influences the incidence rate of CIA [29, 44]. Intestinal microbiotas begin to change before the onset of CIA. Furthermore, the ratio of *Lactobacillus* to *Clostridium* increases during the development of CIA [44, 45], and the abundance of *Lactobacillus* is also increased in patients with RA [46]. In this study, although there was no significant difference in the relative abundance of *Lactobacillus*, the increase in the ratio of *Lactococcus* in the ω-3 PUFA + TPPU group was conspicuous. *Lactococcus* and *Lactobacillus* are lactic acid bacteria, which are known as probiotics. These bacteria affect host health through regulation of the immune system, thereby increasing intestinal barrier function, maintaining intestinal flora balance, and regulating nutrients and

other mechanisms [47]. Both vitamin K and SCFA, which stood out in the present study, are also produced by lactic acid bacteria. Vitamin K plays an important role in arthritis and SCFA in maintaining the intestinal barrier. The SCFA production trend was unclear based on this pathway analysis, but vitamin K-related biosynthetic pathways tended to be enhanced in the ω-3 PUFA + TPPU group and suppressed in the ω-6 PUFA + PEG group. Vitamin K activates the vitamin K-dependent protein, osteocalcin, and therefore, vitamin K2 is an effective drug for osteoporosis [48]. Moreover, vitamin K2 also inhibits the proliferation of the synovial membrane, both *in vitro* and *in vivo* [49]. Vitamin K2 has 11 homologues (MK-*n*; *n* = 4–14), where MK-7 is abundant in natto (fermented soybeans) and was reported to have higher bioactivity than other homologues [50]. Furthermore, oral tablets of MK-7 have been demonstrated to inhibit arthritis activity in RA using a randomized clinical trial [51]. Enhancement of

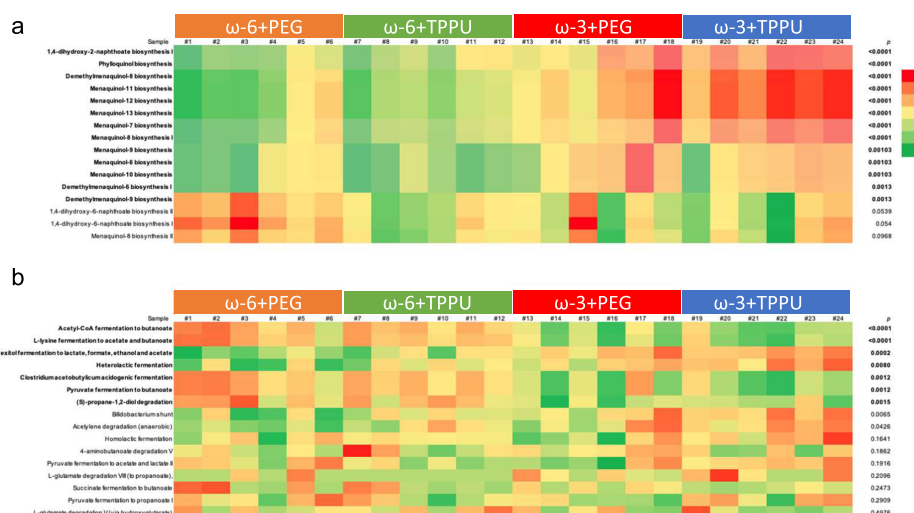


Figure 8. Heatmap of biosynthetic pathways and each treatment group. All pathways, classified into phyloquinol, menaquinol, demethylmenaquinol, and 1,4-dihydroxy-2-naphthoate biosynthesis (a) and fermentation to SCFAs (b), were extracted based on pathway analysis using PICRUST2. The intensity values for each pathway were normalized. Red and green indicate positive and negative correlations, respectively, in the comparison between treatments. The analysis was performed using a two-way ANOVA.

the vitamin K biosynthetic pathway, including MK-7, could cause the suppression of arthritis in the ω -3 PUFA + TPPU group, without suppressing serum IgG and proinflammatory cytokines.

These increases in *Lactococcus* and vitamin K pathways were caused by not only ω -3 PUFA + TPPU direct effect but also dysbiosis-related changes, because of the absence in non-CIA mice. Since the ratio of *Lactococcus* tends to be lower in the CIA mice than in the non-CIA mice except ω -3 PUFA and TPPU group, *Lactococcus* may be one of the indicators of dysbiosis.

In conclusion, combination therapy with ω -3 PUFAs and TPPU markedly suppressed joint inflammation and destruction in CIA. When considering the practical application of sEHi in the future, it may be advisable to consider a fixed combination with ω -3 PUFAs, considering recent lifestyle trends that tend to include a diet rich in ω -6 PUFAs. Changes in the gut microbiota were evident, and the relative increase in *Lactococcus* inversely correlated with the arthritis score, underscoring the need for further investigation of its probiotic effects.

This study had several limitations. First, the exact mechanism by which ω -3 PUFAs and TPPU supplementation suppressed arthritis was not fully elucidated. In the future, we aim to compare the direct effects of ω -6 and ω -3 EpFAs on arthritis. Second, we did not measure the concentrations of EpFAs or vitamin K in any samples due to the technical difficulties. Third, it is unclear whether changes in the gut microbiota are a cause or a consequence of arthritis.

Acknowledgements

We thank the Bioengineering Lab Co. (Japan) and Biopathology Institute Co. (Japan) for technical support. We also thank Editage (www.editage.jp) for English language editing. The graphical abstract was created with BioRender.com.

Supplementary data

Supplementary data is available at *Modern Rheumatology* online.

Conflict of interest

None declared.

Funding

This work was supported by Grants-in-Aid for Scientific Research (grant number 17K10000).

References

- [1] Radu AF, Bungau SG. Management of rheumatoid arthritis: an overview. *Cells* 2021;10:2857.
- [2] Studenic P, Aletaha D, de Wit M *et al.* American College of Rheumatology/EULAR remission criteria for rheumatoid arthritis: 2022 revision. *Ann Rheum Dis* 2023;82:74–80.
- [3] Murakami I, Murakami K, Hashimoto M *et al.* Intake frequency of vegetables or seafoods negatively correlates with disease activity of rheumatoid arthritis. *PLoS One* 2020;15:e0228852.
- [4] Scaioli E, Liverani E, Belluzzi A. The imbalance between n-6/n-3 polyunsaturated fatty acids and inflammatory bowel disease: a comprehensive review and future therapeutic perspectives. *Int J Mol Sci* 2017;18:2619.
- [5] Kim JY, Lim K, Kim KH *et al.* N-3 polyunsaturated fatty acids restore Th17 and Treg balance in collagen antibody-induced arthritis. *PLoS One* 2018;13:e0194331.
- [6] Nelson J, Sjöblom H, Gertsson I *et al.* Do interventions with diet or dietary supplements reduce the disease activity score in rheumatoid arthritis? A systematic review of randomized controlled trials. *Nutrients* 2020;12:2991.
- [7] de Bus I, Witkamp R, Zuillhof H *et al.* The role of n-3 PUFA-derived fatty acid derivatives and their oxygenated metabolites in the modulation of inflammation. *Prostaglandins Other Lipid Mediat* 2019;144:106351.

- [8] Arad M, Waldman M, Abraham NG *et al.* Therapeutic approaches to diabetic cardiomyopathy: targeting the antioxidant pathway. *Prostaglandins Other Lipid Mediat* 2020;150:106454.
- [9] Ghoshal K, Li X, Peng D *et al.* EET analog treatment improves insulin signaling in a genetic mouse model of insulin resistance. *Diabetes* 2022;71:83–92.
- [10] Lai J, Chen C. The role of epoxyeicosatrienoic acids in cardiac remodeling. *Front Physiol* 2021;12:642470.
- [11] Zarriello S, Tuazon JP, Corey S *et al.* Humble beginnings with big goals: small molecule soluble epoxide hydrolase inhibitors for treating CNS disorders. *Prog Neurobiol* 2019;172:23–39.
- [12] Huerta-Yepez S, Tirado-Rodriguez A, Montecillo-Aguado MR *et al.* Aryl hydrocarbon receptor-dependent inductions of omega-3 and omega-6 polyunsaturated fatty acid metabolism act inversely on tumor progression. *Sci Rep* 2020;10:7843.
- [13] Yang Y, Xu X, Wu H *et al.* Differential effects of 17,18-EEQ and 19,20-EDP combined with soluble epoxide hydrolase inhibitor t-TUCB on diet-induced obesity in mice. *Int J Mol Sci* 2021;22:8267.
- [14] Wagner KM, Gomes A, McReynolds CB *et al.* Soluble epoxide hydrolase regulation of lipid mediators limits pain. *Neurotherapeutics* 2020;17:900–16.
- [15] Ren Q, Ma M, Ishima T *et al.* Gene deficiency and pharmacological inhibition of soluble epoxide hydrolase confers resilience to repeated social defeat stress. *Proc Natl Acad Sci USA* 2016;113:E1944–52.
- [16] Hashimoto K. Soluble epoxide hydrolase: a new therapeutic target for depression. *Expert Opin Ther Targets* 2016;20:1149–51.
- [17] Ren Q, Ma M, Yang J *et al.* Soluble epoxide hydrolase plays a key role in the pathogenesis of Parkinson's disease. *Proc Natl Acad Sci USA* 2018;115:E5815–23.
- [18] Griñán-Ferré C, Codony S, Pujol E *et al.* Pharmacological inhibition of soluble epoxide hydrolase as a new therapy for Alzheimer's disease. *Neurotherapeutics* 2020;17:1825–35.
- [19] Codony S, Pont C, Griñán-Ferré C *et al.* Discovery and in vivo proof of concept of a highly potent dual inhibitor of soluble epoxide hydrolase and acetylcholinesterase for the treatment of Alzheimer's disease. *J Med Chem* 2022;65:4909–25.
- [20] Pu Y, Cheng R, Zhang Q *et al.* Role of soluble epoxide hydrolase in the abnormal activation of fibroblast-like synovio-cytes from patients with rheumatoid arthritis. *Clin Immunol* 2023;257:109850.
- [21] Trindade-da-Silva CA, Clemente-Napimoga JT, Abdalla HB *et al.* Soluble epoxide hydrolase inhibitor, TPPU, increases regulatory T cells pathway in an arthritis model. *FASEB J* 2020;34:9074–86.
- [22] Teixeira JM, Abdalla HB, Basting RT *et al.* Peripheral soluble epoxide hydrolase inhibition reduces hypernociception and inflammation in albumin-induced arthritis in temporomandibular joint of rats. *Int Immunopharmacol* 2020;87:106841.
- [23] Maeda Y, Takeda K. Role of gut microbiota in rheumatoid arthritis. *J Clin Med* 2017;6:60.
- [24] Fu Y, Wang Y, Gao H *et al.* Associations among dietary omega-3 polyunsaturated fatty acids, the gut microbiota, and intestinal immunity. *Mediators Inflamm* 2021;2021:8879227.
- [25] Costantini L, Molinari R, Farinon B *et al.* Impact of omega-3 fatty acids on the gut microbiota. *Int J Mol Sci* 2017;18:2645.
- [26] Calder PC. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans* 2017;45:1105–15.
- [27] Pu Y, Zhang Q, Tang Z *et al.* Fecal microbiota transplantation from patients with rheumatoid arthritis causes depression-like behaviors in mice through abnormal T cells activation. *Transl Psychiatry* 2022;12:223.
- [28] Hayer S, Vervoordeldonk MJ, Denis MC *et al.* 'SMASH' recommendations for standardised microscopic arthritis scoring of histological sections from inflammatory arthritis animal models. *Ann Rheum Dis* 2021;80:714–26.
- [29] Segata N, Izard J, Waldron L *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
- [30] Kramer J, Proschak E. Phosphatase activity of soluble epoxide hydrolase. *Prostaglandins Other Lipid Mediat* 2017;133:88–92.
- [31] Huang P. Research progress on the protective mechanism of a novel soluble epoxide hydrolase inhibitor TPPU on ischemic stroke. *Front Neurol* 2023;14:1083972.
- [32] Seymour BJ, Trent B, Allen BE *et al.* Microbiota-dependent indole production stimulates the development of collagen-induced arthritis in mice. *J Clin Invest* 2023;134:e167671.
- [33] Nishida Y, Shirakashi M, Hashii N *et al.* Pathogenicity of IgG-Fc desialylation and its association with Th17 cells in an animal model of systemic lupus erythematosus. *Mod Rheumatol* 2024;34:523–9.
- [34] Buhre JS, Becker M, Ehlers M. IgG subclass and Fc glycosylation shifts are linked to the transition from pre- to inflammatory autoimmune conditions. *Front Immunol* 2022;13:1006939.
- [35] Luo XQ, Duan JX, Yang HH *et al.* Epoxyeicosatrienoic acids inhibit the activation of NLRP3 inflammasome in murine macrophages. *J Cell Physiol* 2020;235:9910–21.
- [36] Murakami T, Nakaminami Y, Takahata Y *et al.* Activation and function of NLRP3 inflammasome in bone and joint-related diseases. *Int J Mol Sci* 2022;23:5365.
- [37] Yamazaki H, Takeoka M, Kitazawa M *et al.* ASC plays a role in the priming phase of the immune response to type II collagen in collagen-induced arthritis. *Rheumatol Int* 2012;32:1625–32.
- [38] Trindade-da-Silva CA, Bettaieb A, Napimoga MH *et al.* Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, rank-related signaling, endoplasmic reticulum stress, and apoptosis. *J Pharmacol Exp Ther* 2017;361:408–16.
- [39] Abshirini M, Ilesanmi-Oyelere BL, Kruger MC. Potential modulatory mechanisms of action by long-chain polyunsaturated fatty acids on bone cell and chondrocyte metabolism. *Prog Lipid Res* 2021;83:101113.
- [40] Gao Y, Feng J, Ma K *et al.* 8,9-Epoxyeicosatrienoic acid inhibits antibody production of B lymphocytes in mice. *PLoS One* 2012;7:e40258.
- [41] Abdalla HB, Puhl L, Rivas CA *et al.* Modulating the sEH/EETs axis restrains specialized proresolving mediator impairment and regulates T cell imbalance in experimental periodontitis. *J Immunol* 2024;212:433–45.
- [42] Tucker L, Trumble TN, Groschen D *et al.* Targeting soluble epoxide hydrolase and cyclooxygenases enhance joint pain control, stimulate collagen synthesis, and protect chondrocytes from cytokine-induced apoptosis. *Front Vet Sci* 2021;8:685824.
- [43] Guo Y, Yu Y, Li H *et al.* Inulin supplementation ameliorates hyperuricemia and modulates gut microbiota in Uox-knockout mice. *Eur J Nutr* 2021;60:2217–30.
- [44] Taneja V. Arthritis susceptibility and the gut microbiome. *FEBS Lett* 2014;588:4244–9.
- [45] Lu C, Chen J, Yi C *et al.* Gut microbiota mediated the protective effects of tuna oil on collagen-induced arthritis in mice. *Food Funct* 2021;12:5387–98.
- [46] Zhang X, Zhang D, Jia H *et al.* The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* 2015;21:895–905.
- [47] Viswanathan VK, Hodges K, Hecht G. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. *Nat Rev Microbiol* 2009;7:110–9.
- [48] Willems BA, Vermeer C, Reutelingsperger CP *et al.* The realm of vitamin K dependent proteins: shifting from coagulation toward calcification. *Mol Nutr Food Res* 2014;58:1620–35.
- [49] Okamoto H, Shidara K, Hoshi D *et al.* Anti-arthritis effects of vitamin K(2) (menaquinone-4)—a new potential therapeutic strategy for rheumatoid arthritis. *FEBS J* 2007;274:4588–94.
- [50] Yamaguchi M, Weitzmann MN. Vitamin K2 stimulates osteoblastogenesis and suppresses osteoclastogenesis by suppressing NF-κB activation. *Int J Mol Med* 2011;27:3–14.
- [51] Abdel-Rahman MS, Alkady EA, Ahmed S. Menaquinone-7 as a novel pharmacological therapy in the treatment of rheumatoid arthritis: a clinical study. *Eur J Pharmacol* 2015;761:273–8.

