Long-chain monounsaturated fatty acids improve endothelial function with altering microbial flora

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JAPAN

Fish oil-derived long-chain monounsaturated fatty acids (LCMUFAs) with a carbon chain length longer than 18 units ameliorate cardiovascular risk in mice. In this study, we investigated whether LCMUFAs could improve endothelial functions in mice and humans. In a double-blind, randomized, placebo-controlled, parallel-group, multi-center study, healthy subjects were randomly assigned to either an LCMUFA oil (saury oil) or a control oil (olive and tuna oils) group. Sixty subjects were enrolled and administrated each oil for 4 weeks. For the animal study, ApoE$^{-/-}$ mice were fed a Western diet supplemented with 3% of either gadoleic acid (C20:1) or cetoleic acid (C22:1) for 12 weeks. Participants from the LCMUFA group showed improvements in endothelial function and a lower trimethylamine-N-oxide level, which is a predictor of coronary artery disease. C20:1 and C22:1 oils significantly improved atherosclerotic lesions and plasma levels of several inflammatory cytokines, including IL-6 and TNF-α. These beneficial effects were consistent with an improvement in the gut microbiota environment, as evident from the decreased ratio of Firmicutes and/ or Bacteroidetes, increase in the abundance of *Akkermansia*, and upregulation of short-chain fatty acid (SCFA)-induced glucagon-like peptide-1 (GLP-1) expression and serum GLP-1 level. These data suggest that LCMUFAs alter the microbiota environment that stimulate the production of SCFAs, resulting in the induction of GLP-1 secretion. Fish oil-derived long-chain monounsaturated fatty acids might thus help to protect against cardiovascular disease. (Translational Research 2021; 237:16–30)

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Abbreviations: LCMUFA = long-chain monounsaturated fatty acid; GLP-1 = Glucagon-like peptide-1; CVD = Cardiovascular disease; NO = nitric oxide; FMD = Flow-mediated dilatation; EPA = eicosapentaenoic acid; SCFAs = Short-chain fatty acids; GPRs = G protein-coupled receptors; TC = total cholesterol; TG = triglyceride; LDL-C = low-density lipoprotein cholesterol; HDL-C = and high-density lipoprotein cholesterol; GC-MS = gas chromatography-mass spectrometry

At A Glance Commentary

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Background

Fish oil from saury is particularly enriched in long-chain monounsaturated fatty acids (LCMUFAs) with at least 20-carbon-long chains (C20 and C22 isomers combined). We have previously shown that saury oil-derived LCMUFAs attenuate atherosclerosis development in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice.

Translational Significance

Participants from the LCMUFA-administrated group in human RCT study showed improvements in endothelial function, increased GLP-1, and a lower trimethylamine-N-oxide (TMAO) levels. Fish oil-derived C20:1 or C22:1 LCMUFAs attenuate atherosclerosis development in ApoE$^{-/-}$ mice with the amelioration of the disturbance in the gut microbiota. Fish oil-derived long-chain monounsaturated fatty acids might thus help to protect against cardiovascular disease.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality and disability worldwide.[1] The early detection of atherosclerotic and/or arteriosclerotic changes in systemic arteries is imperative to prevent the development and/or progression of CVD.[2] Endothelial dysfunction is an early, reversible step in the development of atherosclerosis characterized by a reduction in the bioavailability of nitric oxide (NO).[3,4] The endothelium has vasoprotective effects, including antioxidative, anti-inflammatory, and anti-platelet aggregation functions.[5,6] Risk factors such as smoking, hypertension, diabetes, and dyslipidemia might induce changes in endothelial cells, leading to the generation of oxidative stress and loss of the endothelium's ability to produce NO.[7,8] Flow-mediated dilatation (FMD) is the most established and commonly used non-invasive method to assess endothelial function. FMD, assessed by percent change of brachial artery flow-mediated dilatation after avascularization using ultrasonography, reflects endothelium-dependent and largely NO-mediated arterial function and has been widely recognized as an early surrogate marker for vascular health and CVD.[9]

Inadequate diet could be a critical risk factor for atherosclerosis and CVD.[10] Numerous pieces of evidence have shown that a lack of omega-3 polyunsaturated fatty acids (PUFAs) such as fish oil-derived eicosapentaenoic acid (EPA) contributes to CVD.[11-13] EPA exhibits anti-inflammatory, anti-fibrotic, and cardioprotective properties and improves vascular functions.[14-16] However, fish oil from saury and pollock contains different fatty acids and is particularly enriched in long-chain monounsaturated fatty acids (LCMUFAs) with at least 20-carbon-long chains (gadoleic acid [C20:1] and cetleic acid, [C22:1] isomers combined). Oleic acid (C18:1) is the most famous MUFA abundant in olive oil and is known to decrease the risk of coronary artery disease compared to saturated fatty acids.[17] A previous study revealed the strong inverse association between LCMUFA levels in red blood cells and CVD even after adjusting for the de novo synthesized MUFAs (palmitoleic acid; C16:1 and C18:1), indicating the possible atheroprotective effects of LCMUFAs.[18] We have previously shown that fish oil-derived LCMUFAs attenuate atherosclerosis development in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice.[19] We hypothesized that MUFAs with different carbon chain lengths have different effects on the endothelial and vessel functions.

Recently, studies have highlighted the beneficial changes in the gut microbiota composition in response to omega-3 supplementation.[20] In particular, a decrease in *Faecalibacterium*, often associated with an increase in the Lachnospiraceae family, *Roseburia*, and Bacteroidetes, has been observed.[21] Omega-3 PUFAs are, in general, associated with anti-inflammatory effects, whereas omega-6 PUFAs are known to mediate pro-inflammatory effects owing to the different downstream lipid metabolites that directly affect gut metabolism.[21,22] The gut microbiota plays critical physiological roles in energy extraction from the intestine and in the control of systemic immunity, as well as local intestinal immunity. Disturbances in the microbiota are deeply associated with the development of several diseases such as colitis, inflammatory bowel disease, metabolic disorders, and even cancer.[23-25] Dietary fibers, proteins, and peptides, which escape digestion by host enzymes in the upper gut, are metabolized by the microbiota in the cecum and colon.[26] Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are the major products from

the microbial fermentative activity in the gut[27] and have beneficial effects on multiple organs, and host metabolism through the G protein-coupled receptors GPR41 and GPR43 expressed on enteroendocrine cells, enteric neurons, and enteric leukocytes.[28] SCFAs have also been found to change metabolic profiles in different ways and contribute to the improvement in metabolic syndrome by promoting the secretion of peptide hormones such as peptide YY and GLP-1.[29] However, since we could not obtain feces to directly assess the microbiota or SCFAs in humans, we used Trimethylamine-N-oxide (TMAO) in humans as an indicator of the gut microbiome and GLP-1 as a marker for SCFAs. TMAO concentrations in the human serum are known to be associated with the gut environment and are increased following the consumption of meat-rich food.[30] Here, we investigated the effects of LCMUFAs on the endothelium and serum lipids in humans, the qualitative and quantitative effects of highly purified MUFAs, C20:1 and C22:1, on the endothelium and atherosclerotic lesions in a mouse model of hyperlipidemia, and the association between these anti-atherosclerotic effects and alterations to the microbiota and its metabolites.

MATERIALS AND METHODS

Clinical trial design. This clinical trial was a double-blind, randomized, placebo-controlled study performed at the Tokushima University Graduate School (Tokushima, Japan) from October 2017 to December 2017. This trial was approved by the Committee for Medical Ethics at Tokushima University Hospital, Tokushima, Japan (approval number: 3047) and registered at UMIN Clinical Trials Registry (registration number: UMIN000029181). Written informed consent based on the Declaration of Helsinki was obtained from all participants. Based on sample size determination using the JMP software (SAS Institute Inc, Tokyo, Japan), 60 healthy subjects (20−60 years of age) not using any medications or supplements were recruited for the study. The primary outcome of this study was improvement of vascular endothelial function. As endothelial function is easily altered by lifestyle including smoking, lack of sleep, and ultra-endurance exercise, even healthy young people may have decreased endothelial function.[31,32] Therefore, in this study, we targeted healthy subjects. Subjects consumed the test food regimen daily over a period of 4 weeks and were randomly assigned to either the LCMUFA group or the control (placebo) group. The fatty acid composition of each capsule is listed in Supplementary Table S1. LCMUFA oil was derived from saury, whereas control oil was derived from olive oil and tuna oil. Quality of the control oil, related to the EPA, DPA, and DHA content, equally matched that of LCMUFA oil. To eliminate the effect of EPA, DPA, and DHA on vascular endothelial function, tuna oil containing EPA but less LCMUFA was used. In the control capsule, the required amount of tuna oil was subtracted from 4.2 g, and the rest was olive oil, mainly containing C18: 1 fatty acid, such that the content of C18: 1 n-9 was 47%. Both groups ingested 4.2 g of oil (12 capsules) daily, all at once or at multiple times, after meals. This trial was performed in accordance with the CONSORT 2010 guidelines.[33] Body composition was assessed by bioelectrical impedance analysis (BIA) using a TANITA DC-320 body composition analyzer (TANITA, Tokyo, Japan). BIA measurements were obtained indoors under controlled temperature conditions (23−28˚C) in the morning after overnight fasting. The following measures were recorded: body weight, muscle mass, fat mass, body fat percentage, lean body mass, and total body water. A trained dietitian interviewed the participants to determine the portion size and frequency of food eaten for one week using the established questionnaire FFQg 4.0. We used a record diary that allowed the participants to record the number of supplements they took each day and collected the remaining supplements on the examination day after the intervention to confirm compliance.

Biochemical assessment. Venous blood samples were collected after overnight fasting at baseline and after the intervention. Blood samples were immediately centrifuged at 1,200 × *g* for 10 minutes at 4˚ C to separate the serum or plasma. Measurements of the following biochemical variables were outsourced to SRL, Inc. (Tokyo, Japan): total cholesterol (TC), HDL-C, LDL-C, TG, aspartate transaminase (AST), and alanine transaminase (ALT), blood urea nitrogen, and creatinine. Measurement of the LOX-index was outsourced to NK medico (Tokyo, Japan).

Serum lipid profile analysis in humans. Serum lipids were extracted by direct esterification using the acetyl chloride-methanol method, as previously described.[34] The presence of bioactive components in the extract was evaluated using gas chromatography-mass spectrometry (GC-MS) by multidimensional gas chromatography coupled with mass spectrophotometry (GC-2010 plus Shimadzu, Kyoto, Japan). This machine was equipped with a strong polarity column DB-Wax (30 m × 0.25 mm ID; Agilent, California, USA). High-purity helium was used as the carrier gas and starting and final temperatures were 170˚C and 240˚C, respectively. Heating was carried out at a rate of 1˚C/min and was isothermally constant for 8 minutes. The fatty acid composition was calculated from the area ratio (%) of each fatty acid from total fatty acids.

Measurement of FMD. We measured FMD according to international guidelines[35] and Japanese guidelines from the Vascular Failure Workshop Group using a vascular ultrasound system equipped with an edge-tracking system for 2D imaging and pulsed Doppler flow velocimeter for automatic measurement (UNEXEF; Unex Co. Ltd., Nagoya, Japan). In brief, the diameter of the brachial artery at rest was measured in the cubital region, and the cuff was subsequently inflated to 50 mm Hg above the systolic blood pressure for 5 minutes and then deflated. The diameter at the same point of the artery was continuously monitored, and the maximum dilation from 120 second after deflation was recorded. FMD was calculated as follows: FMD (%) = (maximum diameter − diameter at rest) × 100 / diameter at rest.

Animals and diet. All animal experiments were approved by the institutional animal care and use committee at the University of Tokushima Graduate School (Tokushima, Japan; approval number: T29-11). Animal handling and experiments were performed in accordance with the guidelines provided by the NIH Guide for Care and Use of Laboratory Animals. Male 7-week-old ApoE$^{-/-}$ mice were purchased from Charles River Japan (RRID: IMSR_ARC:APOE, Kanagawa, Japan) and maintained under specific pathogen-free, humidity-controlled conditions at 22 ± 2°C with a 12-hours light/12-hours dark cycle. The mice were divided into three groups according to the type of diet (n = 12; 3.0% control butter group, n = 10; 3.0% (w/w) C20:1, n = 10; and 3.0% (w/w) C22:1). For 12 weeks, ApoE$^{-/-}$ mice were fed a Western diet D12079B, containing 0.15% cholesterol, 21% fat, 50% carbohydrate, and 20% protein (Research Diets, Inc., NJ, USA), supplemented with 3.0% butter, 3.0% (w/w) C20:1 or 3.0% (w/w) C22:1 (Supplemental Table 2) extracted from saury oil, and all of which were iso-caloric. C20:1 fractionated oil and C22:1 fractionated oil were supplied by Nippon Suisan Kaisha (Tokyo, Japan). Unsalted butter was purchased from Megmilk Snow Brand (Tokyo, Japan). The mice were provided water ad libitum. Body weight and food intake of each mouse were measured once per week.

Serum lipid analysis in mice. Hundred-microliter blood samples were collected from the tail veins of all mice every 4 weeks from the start of the experiment. Serum samples were prepared by the centrifugation of the clotted blood at 1,200 × *g* for 15 minutes at 4° C and stored at −80° C until analysis. The following biochemical variables were measured by Oriental Yeast CO., LTD. (Tokyo, Japan): TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Atherosclerotic lesion analysis. At the end of the 12-week feeding period, the atherosclerotic lesions in ApoE$^{-/-}$ mice were determined as previously described.[19] In brief, mice were sacrificed with pentobarbital and perfused via the left ventricle with a 0.9% sodium chloride solution at a constant pressure. The whole aorta was immediately excised and fixed in 10% neutral buffered formalin, washed with phosphate-buffered saline, and stained with a Sudan IV solution. Excess stain was washed off with ethanol. The aorta was longitudinally cut open, and images were captured using an Olympus digital camera mounted on an Olympus dissecting microscope. Lesion area was measured using ImageJ software (National Institutes of Health, MD, USA) and expressed as a percentage of lesion area relative to the area of the whole aorta.

Gene expression analyses. Total RNA was extracted from the abdominal aorta using TriZOL (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed into complementary DNA (cDNA) using the TaKaRa PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). Real-time PCR was performed in 10 μL containing 50 ng of the cDNA template and primers, using StepOnePlus (Applied Biosystems, Carlsbad, CA, USA) and Fast SYBR Green Master Mix (Applied Biosystems). All samples were run in duplicate in a single 96-well reaction plate. Data were analyzed by the ΔCt method. Gene expression was scaled to the expression of the *beta-actin* gene. The primers used are listed in Supplementary Table S3.

Determination of microbiota by 16S rRNA gene sequencing. The murine fecal samples were collected from the caecum after a 12-week-treatment with each oil, placed in tubes, and weighed. Bacterial DNA was extracted using the NucleoSpin DNA Stool kit (TaKaRa Bio Inc, Shiga, Japan) according to the manufacturer's protocol. The 16S ribosomal RNA gene sequencing was performed by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). Briefly, the bacterial 16S ribosomal RNA gene amplicon sequence library was prepared. To analyze the V4 region, the first PCR was performed using Bakt_341F and Bakt_805R primers, and the second amplification was conducted using the index primers.[36] Equimolar amounts of purified DNA amplicons were further amplified on a GridlON X5 platform (Oxford nanopore Technologies, Oxford, UK) for paired-end sequencing (2 × 300 bp) according to the standard protocols presented by Bioengineering Lab Co., Ltd (Kanagawa, Japan). QIIME version 2.0 (2019.4), with the default parameter values, was used for sequence denoising using the DADA2 method for chimera checking.[37,38] The Operational Taxonomic Units (OTUs) table was clustered with a 97% similarity cutoff based on the open-reference approach using UCLUST.

Aorta preparation and vascular reactivity assay. At 8 weeks after feeding, mice were sacrificed by an overdose of pentobarbital. The whole aorta was immediately isolated and the fat and connective tissue around the aorta were carefully excised. Aortic rings of 3 mm were cut from the thoracic aorta for vascular reactivity analysis. Aortic rings were placed in an organ bath MTOB-1Z (Lab Support Co., Ltd., Nagano, Japan) filled with modified Krebs-Henseleit buffer (118.4 mM sodium chloride [NaCl], 4.7 mM potassium chloride [KCl], 2.5 mM calcium chloride [$CaCl_2$], 1.2 mM monopotassium phosphate [KH_2PO_4], 1.2 mM magnesium sulfate [$MgSO_4$], 25 mM sodium bicarbonate [$NaHCO_3$], and 11.1 mM glucose) that was aerated (95% O_2, 5% CO_2) and warmed (37˚C). The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were primed with 31.4 mM KCl and pre-contracted with phenylephrine, producing submaximal (60% of maximum) contraction. Once the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (10^{-9} to 10^{-4} M), sodium prostaglandin F_{2a} (PGF_{2a}; 1.0×10^{-6} to 5.0×10^{-6} M), C20:1 n-11, and C22:1 n-11 (1, 10, and 30 μM) to obtain cumulative concentration−response curves. A PowerLab 8/35 8ch (AD Instruments Japan, Nagoya, Japan) was used for the analysis.

Enzyme-linked immunosorbent assay (ELISA). Venous blood samples were collected after overnight fasting at baseline and after the intervention. Blood samples were centrifuged at 1,200 × *g* for 10 min at 4˚ C to separate the serum. Trimethylamine-N-oxide (TMAO) concentrations in the human serum were determined using an ELISA kit (Glory Science Co., Ltd, Changhua, Taiwan) according to the manufacturer's protocol. Acylated ghrelin levels in human serum were determined using an EIA kit (Bertin Bioreagent, Montigny, le Bretonneux, France) according to the manufacturer's protocol. Both samples were measured for the absorbance using a TECAN Sunrise plate reader (Tecan Japan, Kanagawa, Japan).

Statistical analysis. Data were expressed as mean ± standard deviation (SD). The primary outcome from the clinical data was a change in FMD level. The required sample size was calculated (with FMD as the primary outcome measure) using data from a previous pilot study (unpublished). A total of 36 participants (18 per group) would be required to detect a statistically significant change following supplementation with an effect size of 0.97, with a two-sided significance level (a) or 0.05 and 90% power (b = 0.8). Allowing for a 30% drop-out rate, we aimed to enroll 26 participants per group. Secondary outcome measures included serum TG, LDL-cholesterol, TMAO, and GLP-1. We also measured changes in anthropometric variables (body weight, body mass index (BMI), waist circumference) as potential confounders. All outcome measures were assessed by two-way repeated measures analysis of variance (ANOVA) with a Bonferroni post-hoc comparison to locate significant differences. Normal distribution was confirmed. For animal experiments, multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA. The Tukey-Kramer test was used to determine the pairs that showed significant differences ($P < 0.05$). All statistical analyses and graph creation were performed using the JMP software (SAS Institute Inc, Tokyo, Japan) and PRISM 7 software (GraphPad Software Inc, San Diego, CA, USA).

RESULTS

Clinical characteristics of subjects. We performed a randomized clinical trial to assess the effects of LCMUFA intake. We set up an LCMUFA group fed with saury oil containing LCMUFAs, mainly C20:1 and C22:1 as well as EPA, and a control group treated with a mixture of olive oil and tuna oils (adjusted to the same amount of EPA as that in the LCMUFA group). A total of 54 participants were randomly allocated to the two study arms. Among the 60 recruited participants, six (10.0%) withdrew from the study before the intervention, and four (6.7%) withdrew during the intervention. No significant differences were observed in the proportion of valid completers between the two groups. As shown in Fig. 1, 50 subjects attended the final visit and were assessed. Baseline measurements were performed before randomization. The baseline characteristics of all subjects are shown in Table I. Participants from the two groups were comparable in terms of age, sex, medical history, body weight, BMI, waist circumference, and systolic and diastolic blood pressure. Although we tried recruiting healthy people from 20 to 60 years of age, only 20- to 23-year-old people were enrolled. The mean age was 21.0 ± 3.4 years, and BMI was 21.8 ± 3.1 kg/m^2; seven (11.2%) subjects were male. TC, HDL-C, LDL-C, and other biochemical levels were not different between the LCMUFA group and the control group. None of the subjects had any history of smoking and diseases, including lifestyle-related or metabolic syndromes. Smoking, habitual alcohol drinking, sports activity, and dietary intake of total energy and nutrients did not differ between the two groups. No significant difference was observed between the two groups in anthropometric markers and the dietary intake of nutrients either at baseline or the interventional period (data not shown).

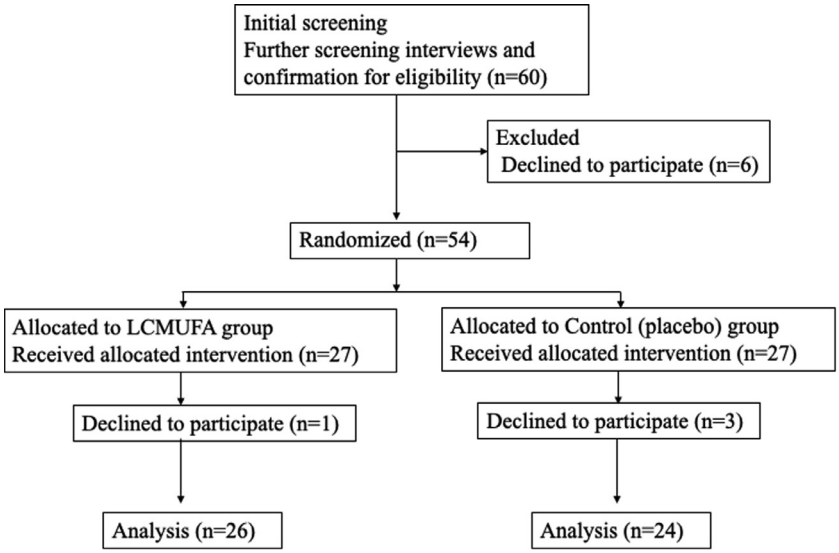

Fig. 1. The flow chart comparing the long-chain monounsaturated fatty acid (LCMUFA) and control (mixture of olive oil and tuna oil) in healthy subjects. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Effects of LCMUFA intake on the glycemic markers and lipid profile in healthy human subjects. The parameters at baseline and after 4 weeks for glycemic markers and lipid profile were comparable between the two study groups, as determined by ANOVA (Table II). As shown in Table II, the level of HDL-C was slightly improved in the LCMUFA group compared to that before the intervention ($P = 0.058$). No significant changes were observed in the fasting glucose and other lipid profiles. Markers of liver and renal functions such as ALT, AST, BUN, and creatinine showed no differences between the two groups.

Table 1. Characteristic of subjects

	Control (n = 24)	LCMUFA (n = 26)	*P*
Demographics			
Age (year)	21.6±0.98	21.5±0.82	*P* = 0.1424
Gender (male/female)	4/24	3/23	
Anthropometrics			
Weight (kg)	55.1±6.8	51.1±14.3	*P* = 0.1269
BMI (kg/m2)	21.5±2.1	21.1±4.0	*P* = 0.1477
body fat (%)	28.7±6.0	26.9±6.7	*P* = 0.2817
Lean body mass (kg)	37.5±6.5	38.75±11.2	*P* = 0.4969
Blood pressures (BP, mmHg)			
Systolic BP	124.5±12.5	124.5±17.9	*P* = 0.884
Diastolic BP	78.5±12.8	805±16.9	*P* = 0.728
Hypertension (>140/90 mmHg)	0	0	n.s
Herat rate	71.2±14.6	71.3±16.2	*P* = 0.895
Blood chemistry			
Total-cholesterol (mg/dL)	171.5±21.6	179.1±31.8	*P* = 0.1291
LDL-cholesterol (mg/dL)	92.1±16.7	97.4±22.1	*P* = 0.1169
HDL-cholesterol (mg/dL)	68.6±10.9	68.6±13.5	*P* = 0.8548
Triglyceride (mg/dL)	55.5±19.0	53.5±20.9	*P* = 0.6516
Deitary assessment			
Energy intake (kcal/day)	1914.0±219.8	1987.5±231.5	*P* = 0.8652
Protein intake (g/day)	64.0±4.9	62.5±5.6	*P* = 0.7682
Fat intake (g/day)	124.0±26.5	128.5±28.3	*P* = 0.9876
n-3/n-6	1/3.85	1/3.87	*P* = 0.7832
Dietary fiber intake (g/day)	18.5±4.9	18.2±4.5	*P* = 0.7565

Table 2. Change of parameter during LCMUFA or control oil intervention

	Control			LCMUFA			Control	LUMUFA	Baseline	4 weeks	Change
	Baseline	4 weeks	Change	Baseline	4 weeks	Change	Baseline vs 4 weeks	Baseline vs 4 weeks	Control vs LCMUFA	Control vs LCMUFA	Control vs LCMUFA
Triglyceride (mg/dL)	55.5±19.0	60.3±23.2	4.4±8.9	53.5±20.9	50.5±19.3	-4.6±10.8	$P = 0.4338$	$P = 0.1332$	$P = 0.6516$	$P = 0.0754$	$P = 0.1236$
Total cholesterol (mg/dL)	171.5±21.6	175.0±24.9	3.5±16.9	179.1±31.8	191.4±32.4	12.4±25.9	$P = 0.6640$	$P = 0.0910$	$P = 0.1291$	$P = 0.0709$	$P = 0.0745$
HDL-C (mg/dL)	68.6±10.9	69.4±13.6	1.33±6.2	68.6±13.5	73.76±17.9	5.2±17.6	$P = 0.8592$	$P = 0.3076$	$P = 0.8548$	$P = 0.3072$	$P = 0.0587$
LDL-C (mg/dL)	92.1±16.7	92.1±20.6	0±12.6	97.4±22.1	99.1±27.6	1.92±20.4	$P = 0.9225$	$P = 0.6085$	$P = 0.1169$	$P = 0.8991$	$P = 0.3789$
LDL-C/HDL-C	1.36±0.29	1.35±0.55	0.0276±0.42	1.51±0.31	1.44±0.53	-0.19±1.03	$P = 0.9665$	$P = 0.4239$	$P = 0.5738$	$P = 0.9596$	$P = 0.1062$
Oxidative LDL (ng/mL)	80.8±23.1	86.7±25.1	5.95±27.2	85.2±24.9	88.5±25.2	3.4±34.5	$P = 0.6055$	$P = 0.9148$	$P = 0.4037$	$P = 0.6727$	$P = 0.5561$
Soluble LOX-1	59.3±12.8	60.4±10.5	0.12±5.6	56.4±13.4	55.3±12.9	-0.08±4.5	$P = 0.5676$	$P = 0.8736$	$P = 0.4768$	$P = 0.4376$	$P = 0.2657$
Fasting glucose (mg/dl)	91.4±5.4	92.1±6.3	0.67±3.8	91.8±5.1	92.0±6.9	0.15±7.9	$P = 0.9019$	$P = 0.4631$	$P = 0.4604$	$P = 0.8517$	$P = 0.3048$
AST (IU/L)	17.6±4.8	17.4±2.7	-0.19±3.8	20.9±4.5	17.7±5.0	-3.29±3.9	$P = 0.7601$	$P = 0.6069$	$P = 0.7601$	$P = 0.4164$	$P = 0.6901$
ALT (IU/L)	14.1±5.1	13.1±3.1	-1.0±6.1	14.0±2.7	13.3±4.0	-0.78±7.4	$P = 0.6024$	$P = 0.8862$	$P = 0.5879$	$P = 0.7921$	$P = 0.7678$
BUN (mg/dL)	13.3±3.4	13.9±3.3	0.64±4.1	13.7±3.3	13.0±3.1	-1.92±3.7	$P = 0.7056$	$P = 0.8437$	$P = 0.5675$	$P = 0.5466$	$P = 0.051$
Creatinine (mg/dL)	0.708±0.12	0.712±±0.11	-0.023±0.21	0.704±0.11	0.7244±0.13	0.0204±0.23	$P = 0.9236$	$P = 0.8657$	$P = 0.7809$	$P = 0.7603$	$P = 0.4227$

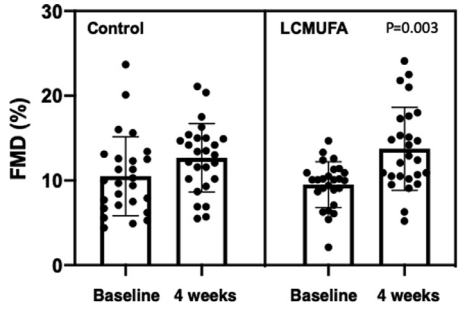

Fig. 2. Change in endothelial-dependent flow-mediated dilatation (FMD) in healthy subjects treated with saury-derived long-chain monounsaturated fatty acid (LCMUFA) oil or control (mixture of olive oil and tuna oil). All data are presented as the mean ± standard deviation. LCMUFA; n = 26, Control; n = 24. Multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

LCMUFA intake improves endothelial function in healthy human subjects. FMD has been the most widely used method to evaluate endothelial function. After 4 weeks of LCMUFA treatment, the ultrasound indices of endothelium functions were found to be comparable between the groups. As shown in Fig. 2, the mean FMD significantly increased from 9.5% to 13.7% ($P = 0.003$) for the LCMUFA group, whereas the value changed from 10.5% to 12.6% for the control group (n.s.).

Serum fatty acid levels in healthy human subjects. As shown in Table III, the serum fatty acid levels were analyzed before and after the intervention. The levels of most fatty acids, including PUFAs, were unchanged after 4 weeks. Gadoleic acid (C20:1 n-11) levels increased by two-fold (0.07 vs 0.14, $P < 0.05$) and cetleic acid (C22:1 n-11) content increased by seven-fold (0.01 vs 0.07, $P < 0.05$) after 4 weeks, whereas no changes were reported in the composition of gondoic acid (C20:1 n-9) and erucic acid (C22:1 n-9). Therefore, we next examined the effect of C20:1 and C22:1 oils on the development of atherosclerosis in ApoE$^{-/-}$ mice.

Plasma lipids, atherosclerosis lesion development, and endothelial dysfunction in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice, a widely used model of atherosclerosis, show increased plasma cholesterol levels and the exacerbated development of extensive atherosclerosis with a Western diet. No difference was observed in the food intake and body weights of ApoE$^{-/-}$ mice fed the Western diets supplemented with 3% C20:1, 3% C22:1, or 3% butter (control) (Supplemental Fig. 1a, b). An analysis of plasma lipids at 4, 8, and 12 weeks revealed no differences in TG, TC, HDL-C, and LDL-C levels among all groups (Supplemental Table S3). The development of atherosclerosis lesions, as assessed from total atherosclerotic lesions, in mice from the LCMUFA groups (both C20:1 and C22:1) was significantly suppressed

Table 3. Percentage of serum fatty acids composition

Fatty acids		Control			LCMUFA		
		Baseline	4 weeks		Baseline	4 weeks	
Lauric acid	12:0	0.00	0.00	n.s.	0.00	0.00	n.s.
Myristic acid	14:0	0.59±0.12	0.47±0.12	$P=0.3647$	0.73±0.25	0.81±0.18	$P=0.5672$
Palmitic acid	16:0	24.4±4.58	23.16±4.26	$P=0.7648$	23.74±4.32	24.15±3.87	$P=0.3128$
Margaric acid	17:0	0.27±0.09	0.25±0.04	$P=0.7628$	0.16±0.04	0.17±0.08	$P=0.7648$
Stearic acid	18:0	10.1±1.98	11.10±1.76	$P=0.4847$	7.81±1.12	8.01±1.87	$P=0.5793$
Arachidic acid	20:0	0.41±0.12	0.45±0.12	$P=0.4389$	0.24±0.07	0.34±0.09	$P=0.7156$
Behenic acid	22:0	0.89±0.12	0.92±0.23	$P=0.3628$	0.48±0.11	0.56±0.99	$P=0.5467$
Tricosylic acid	23:0	0.31±0.08	0.38±0.09	$P=0.8098$	0.16±0.04	0.21±0.03	$P=0.4686$
Lignoceric acid	24:0	0.66±0.11	0.69±0.18	$P=0.4546$	0.41±0.12	0.38±0.07	$P=0.5098$
Total SFA		37.63±5.23	37.42±6.98	$P=0.4723$	33.73±4.82	34.63±5.49	$P=0.3653$
Tetradecanoic acid	14:1n-5	0.00	0.00	n.s.	0.00	0.00	n.s.
Palmitoleic acid	16:1n-7	0.39±0.12	0.51±0.19	$P=0.5463$	0.37±0.09	0.41±0.08	$P=0.6509$
Oleic acid	18:1n-9	15.89±2.34	19.73±2.45	$*P=0.0421$	16.74±2.91	17.66±2.93	$P=0.5926$
Gondoic Acid	20:1n-11	0.06±0.11	0.05±0.08	$P=0.1652$	0.07±0.02	0.14±0.04	$*P=0.037$
Gadoleic acid	20:1n-9	0.17±0.04	0.07±0.01	$P=0.6327$	0.18±0.03	0.16±0.02	$P=0.5638$
Cetoleic acid	22:1n-11	0±0.01	0.01±0.01	$P=0.8726$	0.01±0.02	0.07±0.02	$*P=0.027$
Erucic acid	22:1n-9	0.02±0.002	0.03±0.001	$P=0.5463$	0.03±0.001	0.03±0.001	$P=0.4982$
Tetracosenoic acid	24:1n-9	1.72±0.23	1.79±0.18	$P=0.6564$	1.67±0.43	1.75±0.29	$P=0.7518$
Total MUFA		18.25±2.45	22.19±2.93	$P=0.5721$	19.07±3.11	20.22±3.76	$P=0.1832$
Alpha-linolenic acid	18:3n-3	0.49±0.10	0.52±0.12	$P=0.5563$	0.57±0.11	0.44±0.09	$P=0.5602$
Eicosapentaenoic acid	20:5n-3	1.37±0.26	1.42±0.51	$P=0.0540$	1.13±0.28	1.38±0.32	$P=0.2826$
Docosapentaenoic acid	22:5n-3	0.52±0.12	0.69±0.16	$P=0.6545$	0.42±0.12	0.57±0.11	$P=0.5622$
Docosahexaenoic acid	22:6n-3	4.35±0.08	4.55±0.12	$P=0.7363$	2.56±0.43	2.95±0.24	$P=0.0651$
Linoleic acid	18:2n-6	24.77±3.78	26.53±3.98	$P=0.4537$	28.23±4.34	27.72±3.22	$P=0.7632$
Gamma-linolenic acid	18:3n-6	0.10±0.08	0.11±0.07	$P=0.5437$	0.38±0.56	0.42±0.12	$P=0.6232$
Eicosadienoic acid	20:2n-6	0.16±0.09	0.15±0.08	$P=0.8795$	0.19±0.08	0.16±0.06	$P=0.4655$
Dihomo-gamma-linolenic acid	20:3n-6	1.10±0.23	1.01±0.22	$P=0.6535$	1.02±0.21	1.10±0.15	$P=0.2837$
Arachidonic acid	20:4n-6	6.65±1.01	7.02±1.12	$P=0.6654$	7.41±1.28	6.91±1.21	$P=0.6421$
Total n-3 PUFA		6.73±0.98	7.18+0.89	$P=0.1932$	4.68±1.01	5.34±1.09	$P=0.0821$
Total n-6 PUFA		32.78±2.39	34.82+2.17	$P=0.2928$	37.23±3.12	36.31±3.43	$P=0.7828$
Total PUFA		39.51±3.26	42.00+2.89	$P=0.2193$	41.91±3.96	41.65±4.02	$P=0.7282$

*$P<0.05$

by over 50% ($P < 0.05$) compared to that in mice from the control group (Fig. 3,A-D).

As shown in Fig. 3,E-J, treatment with both C20:1 and C22:1 significantly reduced the mRNA expression of inflammatory molecules such as those encoding chemokines, adhesion molecules, macrophage markers, and cytokines such as monocyte chemoattractant protein-1 (MCP-1), intracellular adhesion molecule (ICAM)-1, CD68, macrophage scavenger receptor (SR-α), and intercellular inflammation (interleukin [IL]-6 and tumor necrosis factor [TNF]-α) in the abdominal aorta ($P < 0.05$) than their levels in the control group, although the two LCMUFA groups showed no difference.

To investigate the mechanism underlying the LCMUFA-mediated attenuation of atherogenesis, we examined their effects on endothelial dysfunction during early atherogenesis. A single administration of LCMUFA resulted in no change in response to acetylcholine in 12-week-old ApoE$^{-/-}$ mice (data not shown). The endothelium-dependent relaxation in response to acetylcholine significantly ameliorated the impaired vasodilation after the continuous 4-week administration of LCMUFA in ApoE$^{-/-}$ mice (Fig. 3, K).

Alterations to the microbiota of the caecum in ApoE$^{-/-}$ mice. We have found that the continuous intake of LCMUFAs improves arteriosclerosis and suppresses inflammation in the abdominal aorta of ApoE$^{-/-}$ mice. Furthermore, LCMUFAs ameliorate the impairment of endothelial function earlier in arteriosclerosis. These benefits were observed only after continuous feeding for 4 weeks or more. Saturated fat modulates the prevalence of dominant bacterial populations within the gut microbiota.[39] However, the effects of LCMUFAs on microbiota have not been examined, and therefore, we next investigated the microbiota composition of the gut using the caecum DNA of those mice from each group by Illumina-based 16S rRNA sequencing. As shown in Fig. 4,A, C20:1 or C22:1 treatment for 12 weeks

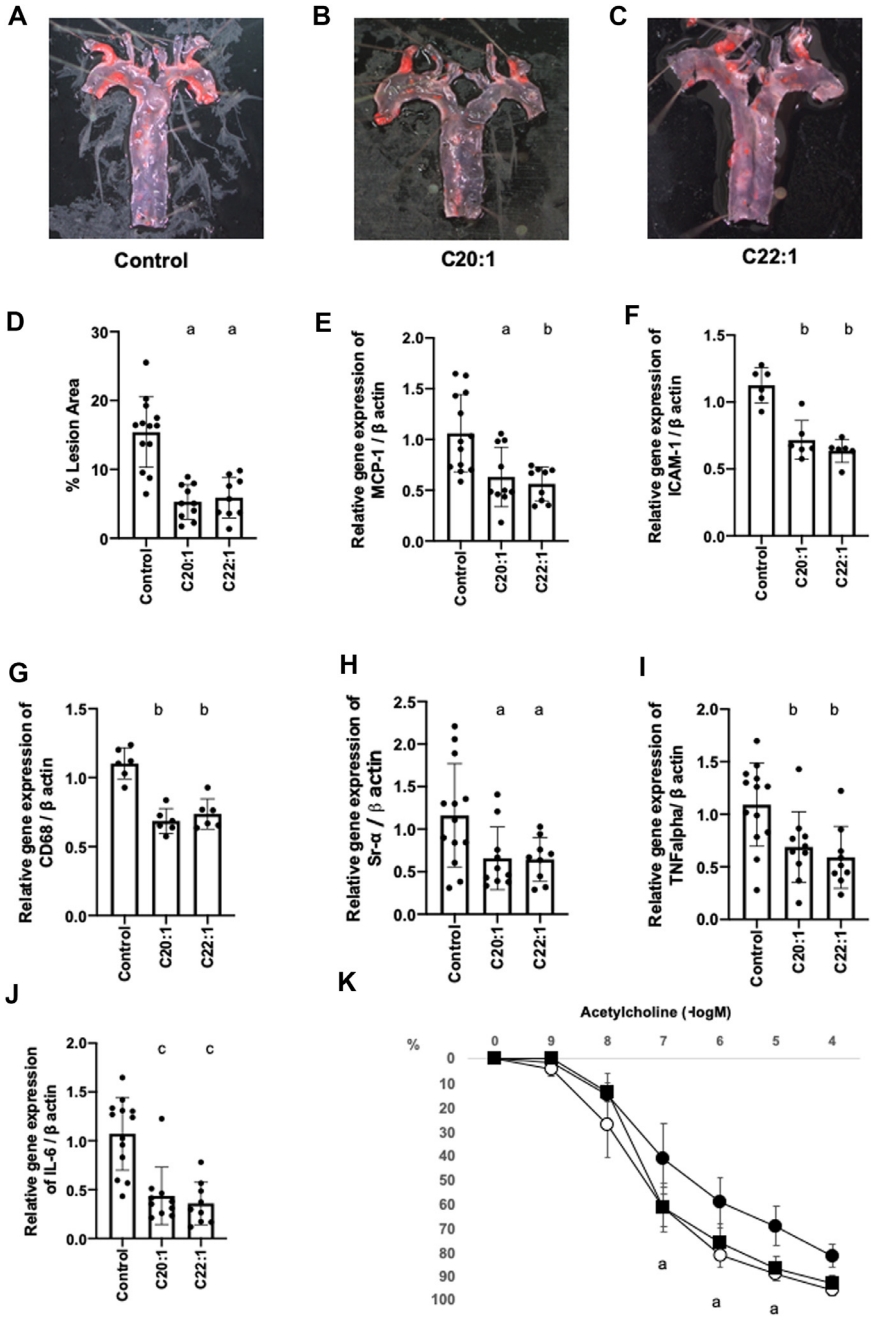

Fig. 3. Development of atherosclerosis in ApoE$^{-/-}$ mice fed a Western diet supplemented with 3% C20:1 or C22:1 for 12 weeks. Representative *en face* Sudan IV staining of aorta (top panels) (**A**−**C**) and quantitative analysis (**D**). (**E**−**J**) Quantitative analysis of relative mRNA expression of genes encoding (**E**) MCP-1, (**F**) ICAM-1, (**I**) CD68, (**H**) Srα, (**I**) TNFα, and (**J**) IL-6 normalized to β-actin in aorta in ApoE$^{-/-}$ mice fed on 3% C20:1 or C22:1 supplemented Western diets. (**K**) C20:1 and C22:1 attenuated endothelial dysfunction in ApoE$^{-/-}$ mice. Vascular reactivity to Ach was determined in ApoE$^{-/-}$ mice; 3% C20:1 or C22:1 with a Western diet intake for 4 weeks ameliorated endothelium-dependent vasodilation in response to Ach compared to that in control chow-fed mice. Number of subjects in different groups were as follows: n = 12; 3.0% control butter group, n = 10; 3.0% (w/w) C20:1, n = 10; 3.0% (w/w) C22:1). Closed circle: control, open circle: C20:1, closed square: C22:1. All data are presented as the mean ± standard deviation. Multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA; a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$; control vs. C20:1- or C22:1-fed ApoE$^{-/-}$ mice. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

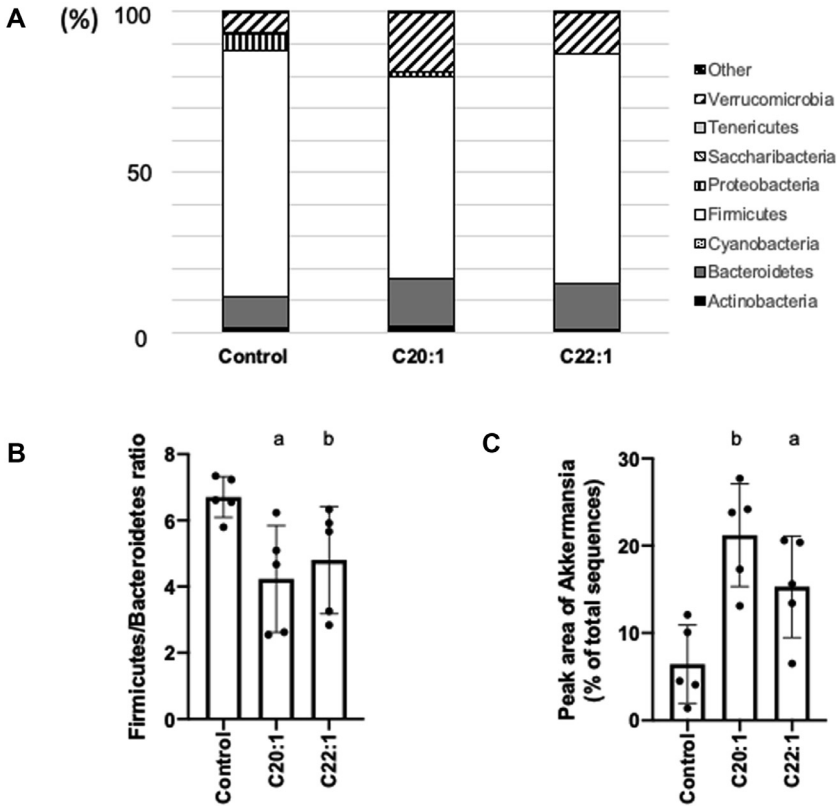

Fig. 4. Fish oil-derived C20:1 and C22:1 alter caecum microbial profiles in ApoE$^{-/-}$ mice fed a Western diet. (**A**) The microbial composition, (**B**) Firmicutes/Bacteroidetes ratio, (**C**) genus level of *Akkermansia* (calculated as a percentage of the total microbiota), and (**D**) microbial composition level of ApoE$^{-/-}$- mice treated for 8 weeks are shown. Values are presented as the mean ± SD. n = 7; 3.0% control butter group, n = 8; 3.0% (w/w) C20:1, n = 8; 3.0% (w/w) C22:1). Multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA; a: $P < 0.05$, b: $P < 0.01$ vs control group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

resulted in alterations to the gut microbiota composition of ApoE$^{-/-}$ mice. The proportion of sequences assigned to Firmicutes significantly decreased in the mice treated with C20:1 or C22:1, whereas the population of Bacteroidetes slightly increased; hence, the ratio of Firmicutes to Bacteroidetes improved in ApoE$^{-/-}$ mice following C20:1 or C22:1 treatment (Fig. 4,B). At the genus level, the sequences assigned to *Akkermansia* were detected in ApoE$^{-/-}$ mice treated with C20:1 or C22:1 (Fig. 4,C). Moreover, the hierarchical clustering analysis of the 35 altered OTUs (as a percentage of total microbiota) revealed separate clusters for the treatment groups at the genus level. Several notable changes were observed at the genus level. In contrast, the proportions of reads assigned to *Clostridium, Lachnospiraceae*, and *Bifidobacterium* were reduced after LCMUFA administration.

LCMUFA intake promotes SCFA and GLP-1 secretion in ApoE$^{-/-}$ mice. The main SCFAs, fecal acetate, propionate, and butyrate, produced by the gut microbiota were assessed. SCFAs markedly increased in C20:1- or C22:1-fed ApoE$^{-/-}$ mice compared to those in butter-supplemented mice ($P < 0.05$ control vs C20:1, $P < 0.05$ control vs C22:1. Fig. 5,A-C). As SCFAs promote plasma GLP-1 production, we also measured GLP-1 levels in the plasma of mice. L cells are located in the epithelial layer of the intestinal mucosa and are responsible for the secretion of the gut hormone GLP-1.[40] As shown in Fig. 5,D, GLP-1 levels were significantly increased after C20:1 or C22:1 treatment ($P < 0.05$ control vs C20:1, $P < 0.05$ control vs C22:1, respectively), which was associated with an increase in GLP-1 mRNA in the colon tissue (Fig. 5,E). GPR43 is a receptor of SCFAs in the L cells and is involved in the induction of GLP-1 expression by SCFAs.[41] GPR43 expression was higher in the colons of C20:1- or C22:1-treated mice compared to that in control mice (Fig. 5,F).

LCMUFA intake improves TMAO and GLP-1 secretion in healthy human subjects. As the gut microbiota and SCFAs clearly changed after LCMUFA treatment in mice, we also assessed some microbiota indicators in

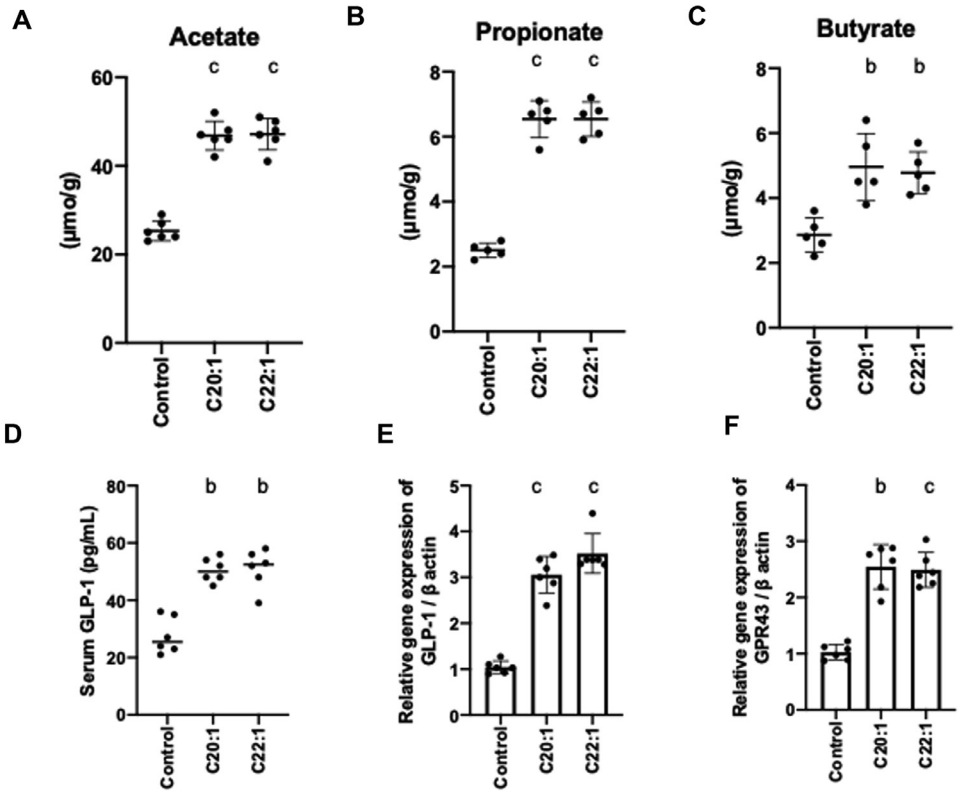

Fig. 5. Fish oil-derived C20:1 and C22:1 improve short-chain fatty acids (SCFAs) in ApoE$^{-/-}$ mice fed a Western diet. (**A**) Acetate, (**B**) propionate, (**C**) butyrate of SCFAs, (**D**) serum GLP-1, (**E**) quantitative analysis of relative mRNA expression of *GLP-1* and (**f**) *GPR43* in colons of ApoE$^{-/-}$ mice treated for 12 weeks; n = 11 per group. Multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA; a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$ versus control group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

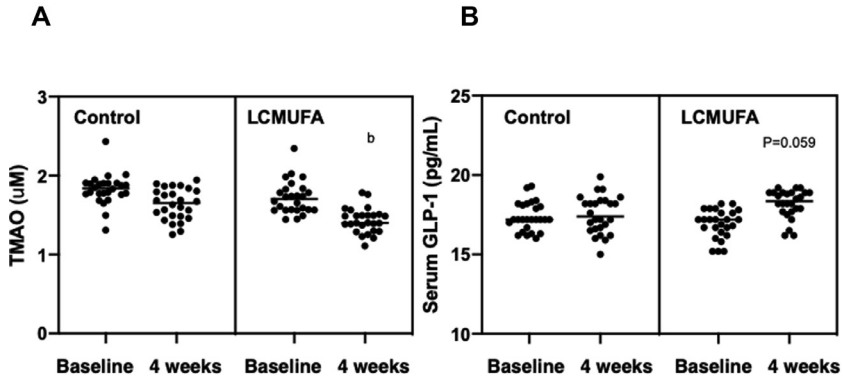

Fig. 6. Change in serum trimethylamine-N-oxide and GLP-1 in healthy subjects. (**A**) Serum trimethylamine-N-oxide (TMAO) and (**B**) serum GLP-1 in healthy subjects administered saury-derived long-chain monounsaturated fatty acids (LCMUFAs) or control oil (mixture of olive oil and tuna oil). Multiple comparisons were performed using the Kruskal-Wallis test and two-way ANOVA; b: $P < 0.01$ versus control olive oil group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

humans; however, we could not obtain feces to directly assess the microbiota or SCFAs. TMAO concentrations in the human serum are known to be associated with the gut environment and are increased following the consumption of meat-rich food.[30] As shown in Fig. 6, A, TMAO levels were significantly decreased in the LCMUFA group after intervention compared to before the intervention ($P = 0.031$), whereas no change was

observed before and after intervention in the control group. We also investigated the level of GLP-1, which is associated with increased SCFAs, and found it to be altered following LCMUFA treatment. The serum level of GLP-1 was not significantly increased in the LCMUFA group ($P = 0.059$) compared to that before the treatment (Fig. 6,B).

DISCUSSION

In the present study, we found that the gut microbial metabolites produced from LCMUFAs alter the host metabolism and alleviate atherosclerosis (Fig. 7). LCMUFAs improved endothelial function not only in mice but also in humans. LCMUFA-enriched fish oil intake contributed to the improvement in the endothelial functions in humans, and both C20:1 and C22:1 fatty acids from LCMUFAs ameliorated atherosclerosis and showed similar effects on the gut microbiota. In addition, we observed that GLP-1 levels were increased through alterations to the microbial environment, consistent with the increase in *Akkermansia* and improvement in the ratio of Firmicutes to Bacteroidetes in mice treated with C20:1 or C22:1, as well as the decrease in TMAO, a CVD prediction marker, in humans treated with LCMUFAs.

Several epidemiology studies and clinical trials have shown that omega-3 PUFAs and a Mediterranean diet including olive oil (C18:1 n-9) can prevent atherosclerotic morbidity, probably through the alleviation of endothelial dysfunction.[42-44] Our clinical study has demonstrated a significant 4.2% increase in FMD following the intake of

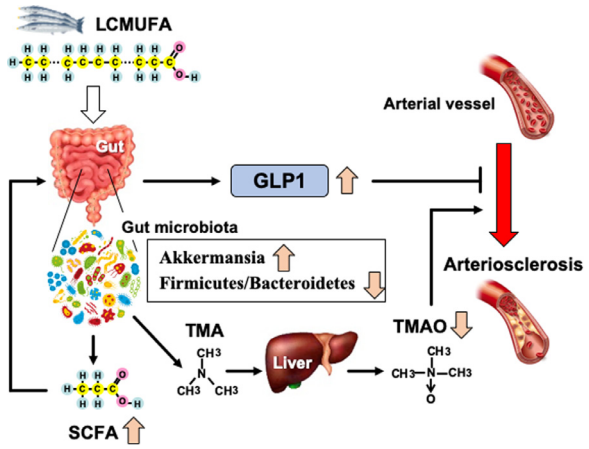

Fig. 7. Schematic model of the mechanism through which saury-derived long-chain monounsaturated fatty acids (LCMUFAs) improve endothelial function via the gut microbiota. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

LCMUFA-rich fish oil for 4 weeks, with a concurrent significant increase in serum gadoleic acid (C20:1 n-11) and cetleic acid (C22:1 n-11) content. More importantly, only a 1% increase in FMD in large trials has been associated with 8−13% reduction in CVD risk.[45,46] Although we could not demonstrate significant changes in blood pressure, lipid profiles, and inflammatory markers, likely due to all values being within a healthy range, HDL cholesterol was slightly increased, consistent with the report by Yang et al.[47] Serum levels of oxidative LDL and the soluble form of LOX-1 (lectin-like oxidized low-density lipoprotein [ox-LDL] receptor-1), a dominant receptor that recognizes and internalizes ox-LDL, as biomarkers of vascular oxidative stress, were also not altered by LCMUFA intake or the control oil (data not shown).[48] Therefore, further studies are needed to elucidate whether LCMUFA can improve dyslipidemia in patients.

To investigate the mechanism underlying the LCMUFA-mediated improvement of endothelial function, we next examined the effect of C20:1 and C22:1 oils in ApoE$^{-/-}$ mice. Both C20:1 and C22:1 oils suppressed the development of atherosclerosis lesions compared to that in mice from the control group, consistent with our previous report about LCMUFA. As we previously reported, pathway analysis results showed that LCMUFAs activate PPAR signaling.[19] We also determined that C20:1 and C22:1 induce the activation of hepatic PPARa and CYP7a1(unpublished data), which modulate cholesterol excretion downstream of the molecule PPAR; however, the serum lipid profiles, including cholesterol, in ApoE$^{-/-}$ mice were not altered by even 12 weeks of intervention. In addition to confirming that fish oil-derived LCMUFA is effective at improving endothelial function in a clinical study, we demonstrated that C20:1 and C22:1 oils improve endothelial function in ApoE$^{-/-}$ mice.

Omega-3 PUFAs prevent atherosclerotic morbidity probably through the alleviation of endothelial dysfunction.[42-44] In addition, an unbalanced omega-6/omega-3 ratio is known to induce metabolic disease and chronic inflammation, and this dysregulation is mediated by the disturbance of the gut microbiota population, which confers host resistance to high-fat diet (HFD)-induced obesity through the production of PUFA metabolites.[49,50] In the present study, we investigated the microbial metabolites produced from LCMUFA intake. Our data on LCMUFAs show that both C20:1 and C22:1 alter the composition of the gut microbiota and mediate anti-inflammatory effects. These include a decrease in the Firmicutes to Bacteroidetes ratio and an increase in *Akkermansia*. Accumulating evidence from animal studies indicates that the interplay among the gut microbiota, omega-3 PUFAs, and immunity helps to maintain the intestinal wall

integrity and the interaction with host immune cells.[22,25] We could not clarify the difference in the mechanism of action between olive oils (C18:1) and LCMUFAs (C20:1 and C22:1), but as demonstrated in this study, it was suggested that the altered microbiota mediated by C20:1 and C22:1 might be the result of improvements in endothelial function. In the present study, we demonstrate that LCMUFA-enriched saury oil improves endothelial functions even in healthy subjects. Our supplement matched the total MUFA and omega-3 fatty acid contents of the control and saury oils; although some serum fatty acid concentrations were altered, the serum EPA concentration was not altered. Therefore, there is a possibility that treatment effects were not only due to LCMUFA but also due to other lower fatty acids. Our supplement included 20% EPA and DHA (840 mg), but the 4-week-intake did not alter the serum fatty acid concentrations. The total composition of EPA and DHA was about 20% of the 4.2 g of supplements taken or about 840 mg. About 1 g or more of EPA is contained in 100 g of tuna, which is similar to the amount ingested by Japanese people every day and the dietary intake on the subjects enrolled in this study. Therefore, it was considered that EPA and DHA are already present in blood lipids and that taking about 840 mg as a supplement does not change their serum lipid concentrations. In fact, 2 g or 4 g EPA daily is prescribed for the drug and to be effective against dyslipidemia. On the contrary, since the serum concentration of LCMUFA was originally low, it is considered that the serum concentration may have increased even with a small amount of supplement.

Our data support the beneficial effects of fish consumption on atherosclerosis, but supplementation with only omega-3 PUFAs might not impart sufficient benefits, and LCMUFA could be one of several compounds in fish oil with beneficial properties.

One of the mechanisms in which the microbiota affects human health and disease is its capacity to produce either harmful metabolites associated with the development of disease or beneficial metabolites that protect against disease. SCFAs produced by the gut microbiota have beneficial effects on host metabolism. SCFAs act through the G protein-coupled receptors GPR41 and GPR43 expressed on enteroendocrine cells, enteric neurons, and enteric leukocytes. These short fatty acids produced by the microbiota change metabolic profiles in different ways. SCFAs can contribute to improvements in metabolic syndrome by promoting the secretion of peptide hormones such as peptide YY and GLP-1.[29] Our data show that LCMUFA intake induces GLP-1 secretion in mice and humans. The decrease in the CVD risk observed after treatment with GLP-1 receptor analogs, liraglutide, and semaglutide, in the LEADED and SUSTAIN-6 trials is thought to be mediated via anti-atherosclerotic mechanisms.[51-53] Nagashima et al. showed that the continuous infusion of native GLP-1 in ApoE$^{-/-}$ mice results in a decrease in the level of atherosclerotic lesions accompanied by a reduction in macrophage infiltration in the vasculature.[54] Exendin-4 also reduces systemic inflammation and improves atherosclerosis, as reported by Wang et al. and Yanay et al.[55,56] Our data suggest that LCMUFAs alter the microbiota environment and promote the production of SCFAs, which induce GLP-1 secretion. GLP-1 thus contributes to the prevention of atherosclerosis.

We failed to investigate the microbiota and SCFAs of humans. However, the level of TMAO, which is the final product of a meta-organismal pathway linking dietary, microbiota, and the host hepatic metabolism and serves as a marker of CVD,[57,58] was improved in subjects treated with LCMUFAs. TMAO promotes upregulation of the expression of multiple macrophage scavenger receptors that are linked with atherosclerosis. As TMAO is derived from dietary phosphatidylcholine, healthy subjects might sometimes exhibit high serum concentrations of this marker.[59] The westernized lifestyle increases phosphatidylcholine and thus TMAO, whereas LCMUFAs from fish improve the TMAO level. Our study with an HFD containing high levels of C20:1 or C22:1 (3%) confirmed the previous results of LCMUFA supplementation, supporting their benefits for endothelial functions and the prevention of atherosclerosis mediated by microbiota.

This study has several limitations. First, we only found favorable changes in the FMD level but not in levels of serum lipids such as LDL-cholesterol. This could be explained by the inclusion of healthy participants who showed normal ranges of serum levels that could not be lowered. In addition, most participants were women, and we could not consider sex differences and sexual cycles. It is also noted that the relatively short duration of our trial might account for the insignificant findings in LDL-cholesterol. In this study, the clinical trial duration was only 4 weeks. Since the primary outcome of this study was FMD improvement, we set a 4-week intervention based on a previous publication by Tong et al.[60] Second, no fecal samples could be analyzed from the participants of the clinical trial. Another limitation is that the dose-response relationship was not tested in our study. In conclusion, our study shows that LCMUFAs can improve endothelial functions and prevent atherosclerosis through alterations to the microbiota-mediated production of SCFAs and GLP-1. Further studies are warranted to confirm these effects in patients with atherosclerosis.

ACKNOWLEDGMENTS

Conflict of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest. The authors have declared that no conflict of interest exists.

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Author contributions are as follow: R.T, H.M, and H.S designed the study. R.T, Y. Y, J.T, M.S, M.B, K.T, Y.M. T, and H.S conducted the clinical studies. J. T, M.B, Y.T, Y.M, N.U, M.K, S.M, N.H, R.Y, and D.F conducted the experimental studies. R.T and M.S analyzed and interpreted data. R.T, J.T, H.M, N.H, K.A, M.S, and H.S oversaw project development. R.T and H.S prepared the manuscript. All authors have read the journal's authorship agreement and that the manuscript has been reviewed by and approved by all named authors.

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DATA STATEMENT

The datasets generated during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.03.016.

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