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No effect of dietary fish oil supplementation on the recruitment of brown and brite adipocytes in mice or humans under thermoneutral conditions

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KEYWORDS

adipose tissue, Ucp1, thermogenesis, obesity, PUFA, n6, n3, fish oil

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ABBREVIATIONS

ADIPOQ	adiponectin	**AUC**	area under the curve
AXOS	arabinoxylan oligosaccharides	**BAT**	brown adipose tissue
CD	control diet	**DHA**	docosahexaenoic acid
EPA	eicosapentaenoic acid	**EET**	epoxyeicosatrienoic acid
HDoHE	hydroxy-DHA	**HEPE**	hydroxy-EPA
HETE	hydroxyeicosatetraenoic acid	**HFD**	high-fat diet
HODE	hydroxyoctadecadienoic acid	**iBAT**	interscapular BAT
IFD	intermediate-fat diet	**iWAT**	inguinal WAT
oxoETE	oxoeicosatetraenoic acid	**scWAT**	subcutaneous white adipose tissue
Ucp1	uncoupling protein 1	**WAT**	white adipose tissue

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ABSTRACT

Scope

Brown and brite adipocytes within the mammalian adipose organ provide non-shivering thermogenesis and thus, have an exceptional capacity to dissipate chemical energy as heat. Polyunsaturated fatty acids of the n3-series, abundant in fish oil, have been repeatedly demonstrated to enhance the recruitment of thermogenic capacity in these cells, consequently affecting body adiposity and glucose tolerance. We scrutinized these effects in mice housed in a thermoneutral environment and in a human dietary intervention trial.

Methods and results

Mice were housed in a thermoneutral environment eliminating the superimposing effect of mild cold-exposure on thermogenic adipocyte recruitment. Dietary fish oil supplementation in two different inbred mouse strains neither affected body mass trajectory nor enhanced the recruitment of brown and brite adipocytes, both in the presence and absence of a β3 adrenoreceptor agonist imitating the effect of cold-exposure on adipocytes. In line with these findings, dietary fish oil supplementation of persons with overweight or obesity failed to recruit thermogenic adipocytes in subcutaneous adipose tissue.

Conclusion

Thus, our data question the hypothesized potential of n3-polyunsaturated fatty acids as modulators of adipocyte-based thermogenesis and energy balance regulation.

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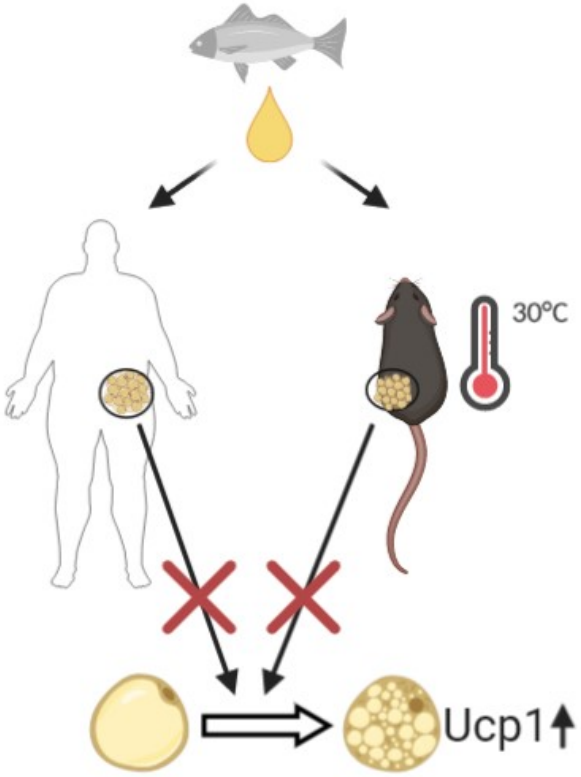

Dietary fish oil supplementation has been repeatedly demonstrated to enhance the recruitment of thermogenic brite cells in subcutaneous adipose tissue. These cells, expressing the uncoupling protein 1 (UCP1), have an exceptional capacity to dissipate chemical energy as heat and are proposed to affect energy balance e.g. reducing body adiposity. In this study, dietary supplementation of fish oil in mice housed at 30°C and humans did not affect the recruitment of UCP1 expressing brite adipocytes.

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Introduction

Brown adipocytes abundantly express uncoupling protein 1 (Ucp1), a protein of the mitochondrial inner membrane that uncouples O2 consumption from ATP production. Activation of Ucp1 enhances macronutrient catabolism, mitochondrial substrate oxidation and consequently, energy expenditure in brown adipose tissue (BAT). The same mechanism is provided by brite adipocytes[1,2], an inducible cell type of white adipose tissue (WAT) that morphologically and functionally resembles bona fide brown adipocytes. The recruitment and activation of thermogenic brown and brite adipocytes is considered a therapeutic strategy to tackle overweight, obesity and associated disorders. In this context, the potential of nutritional components has been comprehensively explored in recent years[3–5].

Polyunsaturated fatty acids (PUFA) influence the effect of a high-fat diet on Ucp1 expression in rodent BAT[6]. Dietary fish oil is a rich source of n3-PUFAs abundantly providing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Supplementation of mice with fish oil or EPA/DHA-enriched diets has been performed repeatedly (for a detailed overview of studies, see Supporting Information Table S1). Multiple studies reported increased Ucp1 expression in BAT and/or WAT upon n3-PUFA supplementation, which can affect body adiposity[7–22], while some reports found no effect on Ucp1 expression [23–27]. Increased Ucp1 expression has been reproduced in cell culture studies employing human and murine models of brown and brite adipogenesis treated with EPA and DHA[14,18,28–31]. Conversely, Ucp1 induction is attenuated or absent upon n6-PUFA supplementation of cultured human adipocytes[20,29,32]. Various direct and indirect mechanisms have been proposed to underlie these observations including interactions of n3-PUFAs with the sympathetic nervous system, an activation of free fatty acid receptor 4, or the metabolization of dietary PUFAs into oxygenated metabolites (oxylipins, i.e. octadecanoids, eicosanoids and docosanoids) of the n6 or n3-series[11,14,20,28,33,34].

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Collectively, the current state of the art suggests a reciprocal effect of n3 and n6-PUFAs on thermogenic adipocyte recruitment and energy balance regulation. However, there are major limitations to this conclusion. Firstly, in vivo studies with mice demonstrating beneficial effects of n3 PUFA supplementation on thermogenic adipocyte recruitment have been mostly conducted under subthermoneutral conditions[7—12,14–19,21–24,26,27,35]. The thermoneutral zone of common laboratory mice is ~30 32°C environmental temperature[36]. Housing of mice below this temperature enforces Ucp1 dependent thermoregulation to maintain body temperature. This process is under control of the sympathetic nervous system which influences the assessment of PUFA effects on thermogenic adipocytes and limits the translational potential to humans, living under thermoneutral conditions most of their lives [37]. Secondly, n3-PUFA supplementation effects are divergent. Studies with mice mostly reported thermogenic adipocyte recruitment in response to high-fat diets comprising fish oil, EPA or DHA instead of lard, palm oil or dairy fat[7–9,11–13,15,16,18], all of which are poor sources for n3-PUFAs. In rats, fish oil supplementation reduces WAT expansion and increases Ucp1 expression in BAT in comparison to safflower oil, which is high in the n6-PUFA linoleic acid[38]. Interestingly, the same effect occurs with borage oil versus safflower oil due to its relatively high content of the linoleic acid-derivative γ linolenic acid[39]. Thus, the effect of borage oil on the recruitment of thermogenic adipocytes may differ from that of other fat sources that are low in n3 PUFAs and/or high in n6 PUFAs. To our knowledge, brown and brite adipocyte recruitment in mice upon fish oil supplementation has not yet been compared to borage oil or any other γ linolenic acid-rich oil. Thirdly, the effect of dietary n3-PUFA supplementation on thermogenic adipocyte recruitment has not yet been investigated in a human dietary intervention trial, leaving the translational relevance of findings from cell culture and rodent in vivo studies unclear.

In this study, we employed two different inbred mouse strains fed with fish oil, borage oil or palm oil under thermoneutral housing conditions to explore the efficacy of dietary n3-PUFA supplementation on body mass accumulation and thermogenic adipocyte recruitment. To elucidate the translational

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potential of this physiology, brite adipogenesis was investigated in subcutaneous adipose tissue of human subjects supplemented with n3-PUFAs during an intervention study. Collectively, our data demonstrate that dietary n3-PUFAs do not affect thermogenic adipocyte recruitment and body adiposity, neither in mice nor in humans.

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1. Material and Methods

Animals and housing

All animal experimentation was performed in accordance with the German animal welfare law with permission from the district government of Upper Bavaria (Regierung von Oberbayern, reference numbers 55.2-1-54-2532-198-13 and ROB-55.2-2532.Vet_02-16-166). An overview of all animal experiments is provided in the supplements (Supporting Information Figure S1). Male mice of the inbred strains C57BL/6J and 129S6/SvEvTac were bred and housed at room temperature (23°C ± 1°C) and fed standard rodent chow-diet (V1124-300, Ssniff Spezialdiäten GmbH, Soest/Germany) prior to the beginning of experiments. At the age of 7-11 weeks, mice were transferred to climate cabinets (HPP750life, Memmert, Schwabach/Germany or UniProtect, Zoonlab, Castrop-Rauxel/Germany) conditioned to 30°C and 55% relative humidity. At the same time, mice were switched to a purified control diet (CD) (S5745-E720, Ssniff) with a fat content of 50 g/kg providing ~13% energy from soybean oil (Supporting Information Table S2). In all experiments, ad libitum CD feeding was conducted prior to other experimental interventions described below. Tissues for molecular analyses were collected from mice killed by CO2 asphyxiation.

Dietary PUFA supplementation of mice

Following 3-4 weeks of CD-feeding, mice were assigned to experimental groups with similar mean body mass. Mice were switched to intermediate-fat diets (IFD) with a total fat content of 140 g/kg providing ~31% energy from soybean oil (50 g/kg) and an experimental fat source (90 g/kg borage oil, fish oil, a proportionate mixture of both, or palm oil; Supporting Information Table S2). Following IFD-feeding, mice were either killed for tissue dissection (Supporting Information Figure S1A and B) or administered high-fat diets (HFD) with a total fat content of 250 g/kg (50 g/kg soybean oil, 110 g/kg palm oil and 90 g/kg experimental oil) providing ~48% energy from fat (Supporting Information Table S2, Supporting Information Figure S1C). Fish-oil comprising diets were produced using a

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pharmaceutical marine oil preparation (Henry Lamotte Oils, Bremen/Germany) with a total n3-PUFA content of ~34% including ~18% EPA and ~12% DHA. All IFDs (S5745-E141 (90 g/kg palm oil), -E142 (90 g/kg borage oil), -E143 (90 g/kg fish oil), -E152 (80 g/kg borage oil, 10 g/kg fish oil), -E153 (60 g/kg borage oil, 30 g/kg fish oil)) and HFDs (S5745-E722 (palm oil), -E146 (borage oil) and -E147 (fish oil)) were produced by Ssniff Spezialdiäten GmbH, and supplemented with butylated hydroxytoluene and exchanged twice per week to minimize fatty acid peroxidation in a thermoneutral environment. Mice were provided ad libitum access to IFDs and HFDs for 4 weeks in all experiments. Food intake was assessed regularly based on the difference in feeder weights between two time points. Body composition was determined by nuclear magnetic resonance spectroscopy (mq7.5, Bruker BioSpin GmbH, Rheinstetten/Germany).

CL-316,243 treatment

Intraperitoneal injection of CL-316,243 (0.2 mg/kg) was conducted once daily on 7 consecutive days at the same time of the day. Vehicle-treated mice received saline. Body composition was assessed on the day of the first injection and on the day after the last injection. Mice were killed and dissected one day after the last injection.

Oral glucose tolerance tests

After 6 hours of fasting, mice received an oral glucose load of 2.8 g/kg lean mass. Blood glucose levels were measured at incised tail tips before (0 min) and during 2 hours after glucose gavage (FreeStyle Lite, Abbott, Wiesbaden/Germany). Fasting and glucose tolerance tests were performed at room temperature (23°C ± 1°C) to enhance glucose uptake by BAT and brite adipose tissue via mild cold-activation. Using this experimental design we previously demonstrated Ucp1-dependent differences in oral glucose tolerance [40]. The total area under the curve of blood glucose levels was

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calculated by the trapezoidal method[41]. Glucose tolerance was determined at the end of IFD and HFD feeding.

Oxylipin analysis

Whole blood was collected in lithium heparin-coated tubes (Sarstedt, Nümbrecht/Germany) and centrifuged for 5 min with 2,000 g at room temperature. The plasma supernatant was transferred to fresh tubes, snap-frozen in liquid nitrogen and stored at -80°C until use. An entire lobe of deep-frozen inguinal WAT was grinded in liquid nitrogen. Aliquots of WAT and plasma were shipped on dry ice to commercial oxylipin analysis (Metatoul platform of metabolomics and fluxomics, Toulouse/France). Oxylipin abundance was normalized to plasma volume or tissue mass, respectively.

Histology

Inguinal WAT and interscapular BAT were fixed in 4% formaldehyde and stored in 70% ethanol. Following automated dehydration and paraffin-embedding, 5 µm sections were drawn to object slides, dried at 37°C and stained with hematoxylin and eosin using an automated multistainer (ST5020, Leica, Wetzlar/Germany). Sections were mounted, dried and analyzed by bright field microscopy (M8, PreciPoint, Freising/Germany).

SDS-PAGE and Western Blot

Interscapular BAT was homogenized with a disperser (Miccra D-1, Miccra GmbH, Heitersheim/Germany) in a total volume of 10 µl/mg lysis buffer (50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitor cocktail (0.1% each, Sigma-Aldrich, St. Louis MO/USA). Homogenates were centrifuged with 14,000-16,000 g at 4°C. The supernatant was centrifuged again and cleared from residual fat. Total protein

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was resolved in a 12.5% gel and transferred to a nitrocellulose membrane. Primary antibodies were applied to detect Ucp1 (~32 kDa, custom-made rabbit-anti-hamster IgG known to reliably detect murine Ucp1[42]) or pan actin (~43 kDa, anti-actin clone c4, Merck Millipore, Burlington MA/USA). A molecular weight marker (PageRuler Prestained Protein Ladder, Thermo Fisher Scientific) was used to confirm the detection of target proteins. IR-dye conjugated secondary antibodies (LI-COR, Lincoln NE/USA) were applied and detected at 700 nm or 800 nm with the Odyssey imager (LI-COR). Image analysis was conducted with the Odyssey software 3.0 (LI-COR) or Image Studio Lite software 5.2 (LI-COR). Ucp1 was normalized to pan-actin or total protein staining (REVERT Total Protein Stain, LI-COR) as indicated in figure legends.

RNA isolation and quantitative real-time PCR of murine samples

Inguinal WAT was homogenized in TRIsure™ (Bioline, London/UK) with a dispersing instrument (Miccra D-1, Miccra, Heitersheim/Germany). Volumes containing precipitated RNA were transferred to spin columns (SV Total RNA Isolation System, Promega, Madison WI/USA), centrifuged for 1 min with 12,000 g and further processed according to the manufacturers protocol. RNA concentrations were determined spectrophotometrically (Infinite 200 PRO NanoQuant, Tecan, Männedorf/Switzerland). Generation of cDNA was performed with the SensiFAST cDNA Synthesis Kit (Bioline) or QuantiTect Reverse Transcription Kit (Qiagen, Hilden/Germany). Quantitative real-time PCR was performed in a 384 well plate format with the LightCylcer 480 system (Roche Diagnostics, Rotkreuz/Switzerland) and SensiMix SYBR no-ROX (Bioline). Primers (Eurofins Genomics Germany GmbH, Ebersberg/Germany): Ucp1 (TCTCTGCCAGGACAGTACCC and AGAAGCCCAATGATGTTCAG), cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (Cidea) (TGCTCTTCTGTATCGCCCAGT and GCCGTGTTAAGGAATCTGCTG), cytochrome c oxidase subunit 7a isoform 1 (Cox7a1) (CCGACAATGACCTCCCAGTA and TGTTTGTCCAAGTCCTCCAA), general transcription factor IIB (Gtf2b) (TGGAGATTTGTCCACCATGA and GAATTGCCAAACTCATCAAAACT),

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ribosomal protein lateral stalk subunit P0 (Rplp0, also known as 36b4) (CTTTATCAGCTGCACATCACTCAGA and TCCAGGCTTTGGGCATCA). Target gene expression was normalized as indicated in figure legends.

Human dietary intervention study

The MyNewGut study was a 12-week randomized crossover trial that investigated diet-induced effects on gut microbiota composition and markers of metabolic syndrome in individuals with overweight[43]. The study is registered at Clinical Trial (NCT02215343), was conducted according to the guidelines laid down in the Declaration of Helsinki, and carried out at the University of Copenhagen (Department of Nutrition, Exercise and Sports) in accordance with the ethical standards of the responsible regional committee on human experimentation in Denmark. In brief, the study was composed of two 4-week dietary interventions separated by a 4-week wash out period. In one of the dietary interventions, participants supplemented their diet with 10.4 g/d wheat bran extract rich in arabinoxylan oligosaccharides (AXOS). In the other dietary intervention, participants supplemented their diet with fish oil containing 3.6 g/d n3 PUFA, including 1.32 g DHA and 1.86 g EPA. Apart from increasing the intake of fish oil, the PUFA intervention also aimed at increasing PUFA intake to 10% of total energy intake by reducing the intake of saturated fatty acids. The participants had not supplemented their diet with fish oil for six weeks preceding study start. Inclusion criteria can be found elsewhere[43]. Briefly, the participants baseline characteristics were; a median age of 46 years (IQR: 34 – 53 years), a median body mass index of 30.0 kg/m2 (IQR: 27.4 – 31.7 kg/m2), a median waist circumference of 94.3 cm (IQR: 90.3 – 102.0 cm). Besides having increased waist circumference 32.1 %, 17.9 %, 7.1 % and 3.6 % of the participants had a metabolic syndrome (MetS) score[44] of 1,2,3 and 4, respectively.

Gene expression analyses in human abdominal subcutaneous adipose tissue

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Subcutaneous adipose tissue was obtained from the abdominal region after an overnight fast before and after each dietary intervention. Following skin sterilization and local anaesthetization (lidocaine 1% or 2%), a small incision was made in the abdominal skin. Trained medical staff sampled ~1 g of subcutaneous fat by biopsy needles (5 mm, Pelomi, Albertslund/Denmark). Adipose samples were immediately rinsed in sterile saline and submerged in Allprotect Tissue Reagent (Qiagen, Hilden/Germany) before being frozen in liquid nitrogen and cryopreserved at -80°C until extraction of RNA. Frozen adipose tissue biopsies (~100 mg) were homogenized using a GentleMACS Dissociater (Miltenyi Biotec, Bergisch Gladbach/Germany). Total RNA was extracted using QIAzol lysis buffer and the miRNeasy Mini Kit (Qiagen). RNA concentration and purity were determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham MA/USA). Synthesis of cDNA was based upon 136 ng of RNA and carried out using TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific) and a 7900 HT Fast Real-Time PCR Thermocycler (Applied Biosystems, Waltham MA/USA). cDNA related to the same participant was synthesized on the same plate. qPCR was performed using a 7900 HT Fast Real-Time PCR Thermocycler (Applied Biosystems), TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), and TaqMan Gene Expression Assay containing primers and probes for Ucp1 (Thermo Fisher Scientific, Hs00222453_m1) and adiponectin (Thermo Fisher Scientific, Hs00605917_m1). All samples from each participant were analyzed in triplicate and on the same plate in order to avoid bias from inter-run variation. Conditions for thermal cycling were: 50°C for 2 minutes, 95°C for 20 minutes, and 50 cycles of 1 second at 95°C and 20 seconds at 60°C. Peptidylprolyl isomerase A (PPIA) (ThermoFisher Scientific, Hs04194521_s1) was chosen as the reference gene for normalization.

Lipidomic Analysis of human plasma

Venous blood samples were drawn after an overnight fasting period at the start and end of each dietary intervention period. Samples were collected in EDTA tubes, placed directly on ice and

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immediately centrifuged at 2500 g for 10 min at 4°C. Samples were stored at -80°C until shipped to University Hospital Regensburg, Germany for lipidomic analysis. Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described previously[45]. A fragment ion of m/z 184 was used for lysophosphatidylcholine[46]. The following neutral losses were applied: PE-based plasmalogens were analyzed according to the principles described by Zemski-Berry[47]. Cholesteryl ester were quantified using a fragment ion of m/z 369 after selective derivatization of free cholesterol[48]. Lipid species were annotated according to the recently published proposal for shorthand notation of lipid structures that are derived from mass spectrometry[49].

Dosage Information

Doses of borage and fish oil supplemented in the mouse diets (Supporting Information Table S2) were chosen in order to match either the n6/n3-ratio found in modern western diets (16:1) or the recommendation for a healthy fatty acid ratio (1:1)[50]. For the diets containing the highest doses of fish oil (90 g/kg) the dose was 225 mg/d based on an average food consumption of 2.5 g/d at 30 g body weight. Based on body surface area[51], this corresponds to a human equivalent fish oil dose of 33.7 g/d for a 60 kg person. This dose of fish oil cannot be achieved by available nutritional supplements.

The MyNewGut study aimed at providing a high amount of n3-PUFA using an intake of six daily capsules (1.32g/d EPA and 1.86 g/d DHA). This amount is at least twice the amount oftentimes applied but is in accordance with EFSAs safety guidelines stating that the combined daily intake of EPA and DHA should not exceed 5 g/d[52].

Statistics

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Data are displayed as individual values with the group mean indicated as horizontal line, or as mean values ± standard deviation. Statistical analyses were conducted using Prism 6 and 8 for mouse and human data, respectively (GraphPad Software Inc., La Jolla CA/USA). Murine data were analyzed by 2-Way ANOVA (Figure 1, Figure 2, Figure 3, Figure 4 E-H, Figure 5 B) or 2-Way repeated measures ANOVA (Figure 4 A-D, Figure 5 A & C-H) and Holm-Sidak post-test if applicable. Significant effects are indicated by asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). Energy intake (Figure 4H, Figure 5B) is depicted as mean energy intake per cage for each mouse. Principle component analysis (PCA) was calculated in R (version 3.6.3) and R-Studio (version 1.2.5033) with the packages factoextra (version 1.0.7) and FactoMineR (version 2.3) on scaled and centered data. Human data were analyzed using two-tailed Wilcoxon matched-pairs signed rank test.

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2. Results

Fish oil and borage oil supplementations cause divergent changes in PUFA metabolism

Dietary fish oil supplementation has been reported to recruit thermogenic adipocytes in murine BAT and WAT [7-22]. To investigate this effect under thermoneutral housing conditions, two common inbred mouse strains, C57BL/6J and 129S6/SvEvTac, were subjected to dietary PUFA supplementation at 30°C (Supporting Information Figure S1A). Mice of both strains received a fish oil-comprising diet rich in EPA and DHA to increase n3 PUFA uptake (dietary n6/n3 ratio = 1.0). Alternatively, mice were administered a borage oil-comprising diet rich in n6-PUFAs including γ-linolenic acid (dietary n6/n3 ratio = 23.2). A third group of mice was used as control and fed an isocaloric, palm oil-comprising diet devoid of these fatty acids (dietary n6/n3 ratio = 12.7; Supporting Information Figure S2). Plasma oxylipin profiling was conducted after 4 weeks of feeding to validate PUFA supplementation by the chosen fat sources. In line with respective elevated PUFA concentrations, fish oil and borage oil increased the total plasma oxylipin concentration compared to palm oil-fed mice of both mouse strains (Figure 1A). The ratio of n6-derived to n3-derived plasma oxylipins was approximatively doubled by borage oil versus palm oil supplementation, while it was strongly reduced by fish oil supplementation (Figure 1B). Thus, borage oil promoted the systemic production of n6 derived oxylipins, while fish oil strongly enhanced n3-derived oxylipin production. This diet-induced difference in oxylipin abundance was less pronounced in inguinal WAT (iWAT), a subcutaneous fat depot with a high propensity to undergo browning. The relative increase in the total oxylipin concentration upon fish and borage oil supplementation was lower in iWAT compared to plasma in both mouse strains and even less pronounced in the 129S6/SvEvTac strain (Figure 1A and C). Similarly, borage oil supplementation doubled the ratio of n6 derived to n3 derived iWAT oxylipins over that of palm oil fed mice in the C57BL/6J but not 129S6/SvEvTac strain, suggesting a strain specific response to n6-PUFA supplementation (Figure 1D). Fish oil supplementation reduced

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the ratio of n6 derived to n3 derived oxylipins in iWAT of both mouse strains (Figure 1B), but within a lower order of magnitude compared to plasma (Figure 1D).

We performed principal component (PC) analyses, which clearly separated the plasma oxylipin profiles of the diet groups (Figure 1E). In line with a strong decrease of the n6/n3 oxylipin ratio in plasma (Figure 1B), fish oil supplementation resulted in a positive shift along PC2 in both mouse strains (Figure 1E). This shift was caused primarily by increased concentrations of n3-oxylipins (Supporting Information Table S3). Additionally, fish oil and borage oil supplementation resulted in a negative shift along PC1, separating both diets from palm oil. This shift was caused primarily by the highly abundant n3-derived hydroxy-DHA (14-HDoHE and 17-HDoHE) and n6-derived hydroxyoctadecadienoic acids (13-HODE and 9-HODE) as well as the n6-oxylipin 6k-PGF1α (Supporting Information Table S3). Similarly, the PC analysis of iWAT separated the oxylipin profile of fish oil-fed mice via a negative shift along PC2 (Figure 1F), based on the contribution of n3-derived oxylipins (Supporting Information Table S4). Oxylipin profile in iWAT of fish oil and borage oil supplemented animals separated from palm oil via a positive shift along PC1 (Figure 1F). This shift was primarily caused by increasing concentrations of n6-derived cytochrome P450 products (5,6-EET, 8,9-EET and 14,15-EET) as well as n6-derived (5-HETE, 5oxoETE and Leukotriene B4) and n3-derived (9-HODE and 13-HODE) lipoxygenase products (Supporting Information Table S4). Interestingly, this shift was more pronounced in iWAT of fish oil-fed C57BL/6J than in 129S6/SvEvTac mice (Figure 1F). Thus, n3-PUFA supplementation via fish oil enhanced the abundance of all detectable n3 derived oxylipins (Supporting Information Tables S3 and S4) but also increased n6 derived oxylipins. Taken together, borage and fish oil comprising diets abundantly provided n6 and n3-PUFAs, respectively, and efficiently modulated fatty acid metabolism.

Dietary n3-PUFA supplementation does not influence the recruitment of thermogenic adipocytes in murine adipose tissues

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To investigate whether dietary n3-PUFA supplementation affected the recruitment of thermogenic adipocytes, we determined Ucp1 expression in BAT and WAT. Administration of n3-rich fish oil did not enhance Ucp1 expression in interscapular BAT (iBAT) or iWAT of either mouse strain (Figure 2A and B). On the contrary, supplementation of n6-rich borage oil increased transcript abundance of brite adipocyte markers Ucp1 and Cidea compared to palm and fish oil-fed mice of the C57BL/6J strain (Figure 2B and C), while the expression of thermogenic marker genes was unaffected by the dietary fat source in WAT of 129S6/SvEvTac mice (Figure 2B-D).

In a second experiment we investigated whether the recruitment of brown and brite adipocytes is facilitated by the availability of n3-PUFAs upon adrenergic stimulation (Supporting Information Figure S1B). C57BL/6J mice were fed diets (Supporting Information Figure S4) with gradual increasing dietary n6/n3 ratios from ~1 (IFD1, previously “fish oil”), ~5 (IFD5), ~11 (IFD11) and ~18 (IFD18, previously “borage oil”) for 4 weeks. Additionally, the β3-adrenoreceptor agonist CL-316,243 was administered on 7 consecutive days during the last week (days 21-28) of the dietary intervention to stimulate the recruitment of brown and brite adipocytes.

In line with the ability to recruit and activate thermogenesis[53,54], CL 316,243 treatment resulted in Ucp1 protein induction and a strong reduction in iBAT mass (Figure 3A and B), the latter of which was likely the consequence of concomitant Ucp1 activation and lipid oxidation to fuel thermogenesis. Similarly, CL 316,243 administration resulted in a slight reduction of iWAT mass (Figure 3C) and the induction of brite adipocyte marker gene expression (Figure 3D-F). In line with these effects, CL 316,243 reversed diet induced lipid droplet hypertrophy in brown adipocytes and promoted iWAT remodeling by brite adipogenesis (Figure 3G). However, the dietary n6/n3 ratio neither affected thermogenic adipocyte abundance in vehicle nor in CL-316,243 treated mice. Interestingly, elevated Ucp1 expression in iWAT of borage oil (IDF18) versus fish oil (IDF1)-fed mice (as observed in our previous experiment, Figure 2B) was absent in mice of this experiment (Figure 3D), suggesting that this effect is not robust. Based on the low gene expression levels of TNFα and

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F4/80 in inguinal white adipose tissue (Supporting Information Figure S6) we did not see any indications of inflammation. Inhibition of Ucp1 gene expression by adipose tissue inflammation and macrophage infiltration as published before [55] is therefore unlikely. Collectively, the recruitment of brown and brite adipocytes was affected by CL 316,243 but not by the dietary fat source.

Mice of all diet groups had comparable body mass and body composition at the beginning of CL 316,243 treatment following 3 weeks of PUFA supplementation (Supporting Information Figure S7). While body mass remained constant or tended to increase in vehicle treated mice, body mass was reduced by CL-316,243 administration initially but remained constant thereafter (Figure 4A-D). This small, CL 316,243-induced body mass reduction was similar for all dietary n6/n3 ratios (Figure 4E) and caused by a reduction in fat, not lean mass (Figure 4F and G). Mice fed with n3-rich IFD1 tended to show attenuated total body mass reduction in response to CL-316,243 despite significant fat mass loss (Figure 4E and F) caused by a minor gain in lean mass (Figure 4G). In line with these physiological effects, energy intake was not influenced by dietary n6/n3 ratios and comparable between vehicle and CL 316,243 treated mice (Figure 4H). Thus, thermogenic adipocyte recruitment and energy balance regulation were influenced by β3 adrenoreceptor agonism but not by dietary fat source. Taken together, dietary fatty acid composition of isocaloric diets fed under thermoneutral housing conditions did not influence recruitment of murine thermogenic adipocytes, neither alone nor in synergy with the β3-adrenergic route of thermogenesis.

Dietary n3-PUFA supplementation does not influence the development of diet-induced obesity

The induction of thermogenic adipocytes by dietary n3-PUFAs has been associated with attenuated development of diet-induced obesity and glucose intolerance[7,8,10,12,15,35,56]. We assessed dietary fat source effects on these parameters in a third experiment (Supporting Information Figure S1C). Mice of the C57BL/6J strain received palm oil, fish oil or borage oil comprising intermediate-fat diets (IFD, 140 g/kg total fat content) as conducted in the previous experiments. In line with our previous

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findings (Figure 4E), body mass gain was unaffected by the dietary fat source during 4 weeks of IFD-feeding (Figure 5A). To promote the development of diet-induced obesity and glucose intolerance, IFDs were subsequently exchanged for high-fat diets (HFD) with a total fat content of 250 g/kg. The original proportion of borage and fish oil (90 g/kg) was maintained in these HFDs (Supporting Information Table S2). As expected, the enhanced caloric value of the HFD promoted body mass gain due to increased energy intake (Figure 5A and B). This enhanced body mass gain during HFD feeding was caused by fat mass, not lean mass gain (Figure 5C E). However, none of these parameters was affected by dietary fatty acid composition. We observed a similar pattern in a parallel experiment conducted with mice of the 129S6/SvEvTac strain (Supporting Information Figure S8A-E).

To elucidate the effect of dietary PUFA supplementation on glucose homeostasis, mice were subjected to oral glucose tolerance tests at the end of IFD and HFD-feeding, respectively. In both mouse strains, blood glucose response after oral glucose gavage was not influenced by the dietary fat source at the end of IFD feeding (Figure 5F and H and Supporting Information Figure S8F and H). In line with enhanced body mass gain, the switch from IFD to HFD impaired glucose tolerance in C57BL/6J mice. Interestingly, this diet induced alteration was prevented by supplementation of fish oil-comprising HFD (Figure 5G and H). Impairment of glucose tolerance by HFD was, however, absent in 129S6/SvEvTac mice (Supporting Information Figure S8G and H) and thus, demonstrating a strain specific protective effect of fish oil.

In summary, dietary n3-PUFA supplementation under thermoneutral conditions did not influence body mass accretion or adiposity in mice, but attenuated diet-induced impairment of oral glucose tolerance in a strain-specific manner.

Dietary n3-PUFA supplementation does not promote browning of WAT in human subjects

Supplementation of n3-PUFAs affects brite adipogenesis of cultured human adipocytes[20,29,30]. To investigate whether such in vitro effects translate into WAT browning in vivo, we took advantage of

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the MyNewGut study, a 12-week randomized crossover trial investigating the effects of a dietary intervention on subjects with overweight and obesity[43]. Twenty-nine participants supplemented their diet either with a wheat bran extract rich in arabinoxylan oligosaccharides (AXOS intervention) or fish oil to consume EPA and DHA at a dose of 3.6 g per day (PUFA intervention). Lipidomic analysis of plasma samples revealed increased levels of cholesteryl esters and other lipids derived from DHA and EPA in response to PUFA but not AXOS supplementation, thus substantiating the efficacy of the dietary intervention (Figure 6A-D, Supporting Information Figure S9). The effect of PUFA supplementation on browning of WAT was investigated based on subcutaneous fat biopsies. As expected, mRNA encoding the adipose-derived hormone adiponectin was abundantly expressed (average Ct-values of 23) confirming the biopsies as adipose tissue (Figure 6E and F). In contrast, Ucp1 mRNA was barely expressed (average Ct-values of 38) and only detectable in a subset of biopsies obtained prior to the start of dietary interventions (Figure 6G and H). Both the PUFA and the AXOS intervention failed to enhance this very low Ucp1 expression (Figure 6G and H). Again, the inhibitory effect of inflammation on Ucp1 gene expression could be excluded based on the lack of effect of fish oil supplementation on systemic inflammation markers (high sensitive C-reactive protein and white blood cell count, as previously published [43], and the stable expression of adiponectin as an anti-inflammatory marker before and after PUFA supplementation (Figure 6F). Thus, dietary n3-PUFA supplementation did not affect WAT browning in humans in vivo.

3. Discussion

Global obesity prevalence has dramatically increased over the last decades entailing a pronounced risk for associated health burdens[57,58]. The potential for intervention by nutritional compounds has been comprehensively explored and a number of candidates with anti-obesogenic potential has been identified[59–62]. Among these are PUFAs of the n3-series[63], rendering dietary fat quality a potential modulator of body mass development. Indeed, the proportionate consumption of n3

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PUFAs has decreased in modern societies[50], suggesting the effect of energy dense food on adiposity to be exacerbated by the excessive intake of other types of fatty acids. Conversely, an increase in dietary n3 PUFA uptake has been hypothesized to counteract this effect by the ability of n3-PUFAs to affect the recruitment of brown and brite adipocytes within the mammalian adipose organ via several different mechanisms[33,64–66]. In this study, we rigorously tested this hypothesis in mice and man.

Previous studies mostly reported brown and/or brite adipocyte recruitment upon dietary n3-PUFA supplementation in mice kept under subthermoneutral conditions [7-12,14–19,21–24,26,27,35,57]. Ambient temperatures below 30°C have a considerable influence on thermogenic adipocyte recruitment as small rodents rely on Ucp1 to maintain their body temperature[67]. This regulation is mediated via the sympathetic nervous system. Importantly, the adrenergic influence on human BAT must be assumed minimal due to clothing and heating systems minimizing heat loss. In mice, dietary n3 PUFAs have been reported to directly influence the activity of the sympathetic nervous system via activation of gastrointestinal afferent pathways, and to synergize the effect of adrenergic stimulation on the recruitment of brown and brite adipocytes[11,14,20,22]. To investigate the significance of adrenergic stimulation, our mice were housed under thermoneutral conditions (i.e. at 30°C) and subjected to β3-agonist treatment during dietary PUFA supplementation. Brown and brite adipocyte recruitment were, in the presence and absence of β3-agonism, not affected by dietary n3-PUFA supplementation in two different mouse strains. Thus, our findings, together with a previous study[25], suggest thermoneutral housing temperature to impede the effect of n3-PUFAs on thermogenic adipocytes. However, our results do not corroborate previous findings demonstrating enhanced recruitment of brown and brite adipocytes upon adrenergic stimulation in mice receiving an n3-PUFA supplemented diet[11,14,20,22].

Interestingly, borage oil supplementation upregulated Ucp1 transcript abundance in C57BL/6J mice in one experiment but not in a second, suggesting an influence by changes in the experimental

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framework including different food batches. Obviously, effects of dietary PUFA supplementation are, in the presence and absence of adrenergic stimulation, insufficiently robust and easily superimposed by confounding factors of experimental setup.

Oxylipin profiling of WAT and plasma revealed profound changes in systemic fatty acid metabolism, corroborating the efficacy of dietary n3 PUFA supplementation in our study. The absence of brite adipocytes in WAT of fish oil-fed mice has recently been associated with n3-PUFA-derived oxylipins formed by non-enzymatic reactions that accumulate in WAT due to lipid peroxidation of fish oil supplemented foods[35]. Indeed, levels of 18 hydroxy-EPA (18-HEPE, an EPA-derived n3-peroxide) were increased in both plasma and WAT of our fish oil-fed mice. Moreover, the ability of dietary n3 PUFAs to recruit brown and brite adipocytes has been associated with a local downregulation of inhibitory, n6-derived oxylipins[20]. In fact, the n6-derived oxylipins 9 HODE and 13-HODE were very abundant in WAT of n3-PUFA supplemented, fish oil fed mice. Interestingly, 9 HODE and 13-HODE enhance the recruitment of brite cells in vitro[68]. However, our own findings point towards a function for these two metabolites as negative regulators of thermogenic adipocytes in vivo[69], possibly due to their function as ligands for peroxisome proliferator-activated receptor γ, the master regulator of adipogenesis[70]. Thus, high WAT levels of 9-HODE, 13 HODE and 18-HEPE may at least partially explain the inability of n3-PUFA supplemented diets to recruit brown and brite adipocytes in mice of our study.

In rodents, fish oil has been reported to have beneficial metabolic effects and to protect against the development of diet-induced obesity[71–73]. Several studies reported a remarkable impact of n3 PUFA supplementation during HFD-feeding leading to significantly lower body mass during the entire feeding period in comparison to mice fed isocaloric control diets[7,10,12]. In an independent study conducted in our facility, small reductions in body mass became significant after 12 weeks of feeding a HFD that contained fish oil instead of palm oil[15]. This effect was absent in the present study. Interestingly, the overall composition of our HFDs was highly similar to the HFDs used by Ludwig and

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coworkers[15], suggesting differences in study design (consecutive IFD and HFD-feeding versus continuous HFD-feeding), feeding duration (n3-PUFA supplementation during 8 weeks versus 12 weeks) and the source of n3-PUFAs (marine fish oil preparation with EPA>DHA versus EPAX concentrate with DHA>EPA) as possible confounders. We question a major influence of the fat source since n3-PUFA-comprising HFDs affect body adiposity and thermogenic adipocyte recruitment irrespective of EPA and DHA abundance[11,18].Further, inappropriate dosage of n3-PUFA can be excluded as a factor influencing our results, as the amount of n3-PUFA (90 mg/kg fish oil including ~20 mg/kg total n3-PUFA) administered are well in line with previously used doses of fish oil (mean dose 108 mg/kg[7–11,25]) and isolated n3-PUFA (mean dose 20 mg/kg[12,13,15,18,20,21]). In line with our findings, dietary n3-PUFA supplementation of mice at thermoneutral housing temperature and in the absence of CL 316,243 reportedly failed to attenuate body mass gain, or required extremely long feeding duration[20,25,56]. Thus, the housing temperature of mice seems to be one crucial confounder influencing the response to dietary n3-PUFA supplementation. Moreover, attenuated body mass gain upon n3-PUFA supplementation even occurs in Ucp1-ablated mice[7,25], suggesting a possible, causal contribution by BAT and WAT via means of other mechanisms such as lipid cycling rather than Ucp1-dependent thermogenesis[16,74]. In conclusion, the protective effect of n3-PUFA supplementation on diet induced obesity is abrogated by thermoneutral housing and not influenced by adipocyte-based, Ucp1-dependent thermogenesis.

Mice of the C57BL/6J strain display a high propensity to develop glucose intolerance[75]. In our study, these mice were protected from HFD-induced glucose intolerance when supplemented with fish oil. A similar protection has been observed in Ucp1-ablated mice[7,25]. Although the recruitment of Ucp1-independent thermogenic mechanisms can account for increased postprandial glucose disposal[76], we speculate that glucose tolerance improvements are more likely to have a different origin. In fact, n3 PUFAs can affect insulin release and insulin sensitivity via various mechanisms, cumulating in an improvement of whole-body glucose tolerance[77,78]. Of note, the translational significance of such

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beneficial fish oil supplementation effects on glucose tolerance, as frequently observed in rodents, seems to have limited relevance for humans[78,79].

While a number of studies has investigated effects in rodents in vivo, the thermogenic recruitment of human adipocytes upon n3-PUFA supplementation has to date only been demonstrated in cultured cells. To overcome this limitation, we took advantage of abdominal subcutaneous adipose tissue specimens collected during a human intervention study assessing effects of dietary n3-PUFA supplementation in persons with overweight and obesity[43]. Importantly, EPA-treatment can increase the expression of thermogenic marker genes in cultured adipocytes from subcutaneous adipose tissue of overweight subjects[30]. Moreover, human subcutaneous adipocytes have a browning potential in vivo[80–86]. In agreement with several other human interventions[87–90], our gene expression data show that Ucp1 transcripts are barely detectable in subcutaneous abdominal fat. Fully in line with our experiments in mice, fish oil supplementation of the study participants over the course of 4 weeks did not at all affect this very low Ucp1 expression, suggesting that an increased intake of n3-PUFAs for one month do not promote WAT browning in humans in vivo, at least in overweight subjects. In line with this observation, resting energy expenditure of the same subjects was not affected by fish oil supplementation[43]. This human intervention study has several limitations. First, it is important to emphasize that changes in adipose gene expression were part of the exploratory outcomes of the MyNewGut study. Secondly, we cannot exclude that four weeks of fish oil supplementation is insufficient for inducing browning of human subcutaneous WAT. Yet, previous studies have demonstrated that this fat depot in humans has the capacity to undergo browning in vivo and suggests that this might occur even in response to acute cold exposure[84,85,91]. Moreover, adrenergic stimuli and phosphodiesterase inhibition can promote Ucp1 expression and brite adipogenesis in human subcutaneous white fat within 1-4 weeks[92–94]. Taken together with our human lipidomics data and the high dose of n3-PUFA applied in the MyNewGut study (3.6 g/d), these observations suggest that fish oil, in physiologically relevant doses, does not promote

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browning of abdominal subcutaneous white fat of subjects with overweight. Fish oil supplementation, however, may stimulate adipose browning in other anatomical areas. Given that body adiposity is associated with an impaired browning capacity [85,95,96], fish oil supplementation may also be hypothesized to be more efficient at inducing browning in lean individuals.

In conclusion, dietary n3-PUFA supplementation did not affect thermogenic adipocyte recruitment or body mass accretion, neither in mice nor in humans. We thus propose disregarding dietary fish oil and n3-PUFAs as translationally relevant agents to modulate energy balance regulation via adipocyte based thermogenesis.

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Author Contributions

SFM conceived the mouse study, planned and performed mouse experiments, conducted molecular analyses on mouse tissues, analyzed and interpreted data, and wrote the manuscript. SD planned and performed mouse experiments, conducted molecular analyses on mouse tissues, analyzed data, contributed to interpretation of the data, and edited the manuscript. JL formulated the human hypothesis, performed qPCR on human tissues, analyzed and interpreted human gene expression and lipidomics data, and edited the manuscript. ALH performed qPCR on human tissues and contributed to the analysis and interpretation of human gene expression data. TF analyzed data and edited the manuscript. CC assisted during the performance of mouse experiments. LK designed and conducted the human clinical study. AA obtained funding for the human clinical study and contributed to study design. MPG contributed to the interpretation of human gene expression data. LHL contributed to human clinical study design and acquisition, and the analysis and interpretation of human gene expression data. GL generated and contributed to interpretation of the lipidomics data set. EZA acquired funding, assisted during the performance of mouse experiments and edited

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the manuscript. MK acquired funding, conceived the mouse study and edited the manuscript. All authors approved the final version of the manuscript.

Competing Interests Statement

The authors declare no competing interests.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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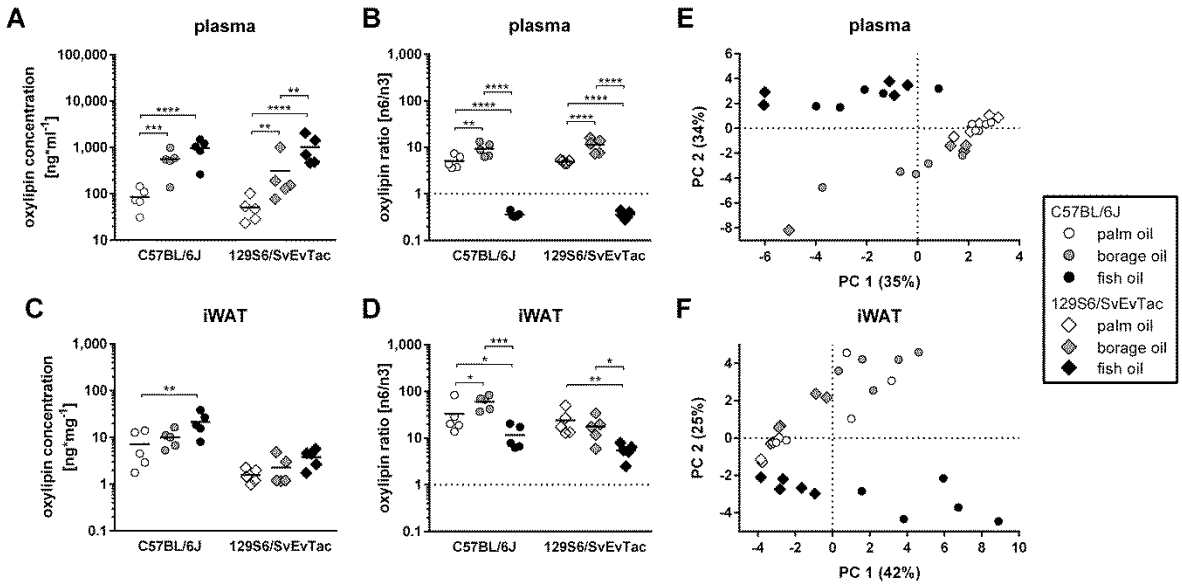

Figure Legends

Figure 1: Effect of dietary PUFA supplementation on the oxylipin profile of plasma and iWAT in C57BL/6J and 129S6/SvEvTac mice. (A) Total concentration of all measured oxylipins in plasma. (B) Ratio of n6-derived to n3-derived oxylipins in plasma. (C) Total concentration of all measured oxylipins in iWAT. (D) Ratio of n6-derived to n3-derived oxylipins in iWAT. (E) Principal component analysis of the plasma oxylipin profile. (F) Principal component analysis of the iWAT oxylipin profile. Asterisks indicate a significant effect of the fat source in C57BL/6J or 129S6/SvEvTac mice (n=5).

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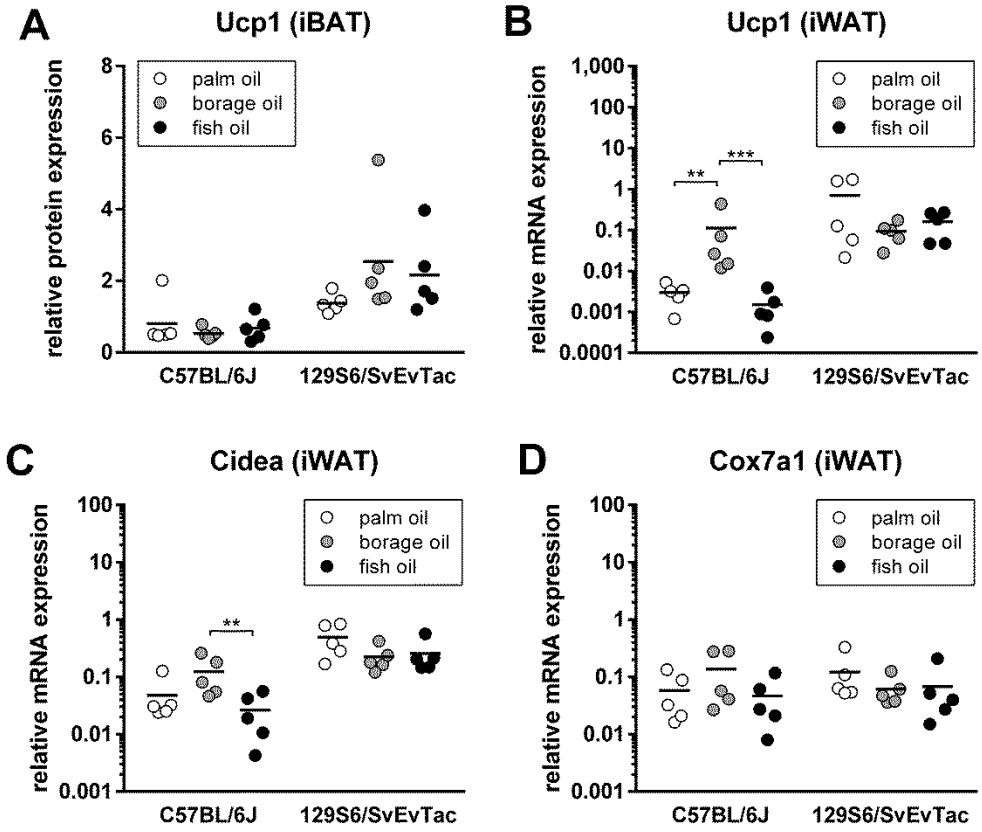

Figure 2: Effect of dietary PUFA supplementation on thermogenic adipocyte recruitment. (A) Relative Ucp1 protein levels in iBAT. Ucp1 was normalized to pan-actin. A representative Western Blot image is displayed in Supporting Information Figure S3. (B-D) Relative gene expression of (B) Ucp1, (C) Cidea and (D) Cox7a1 in iWAT. Gene expression was normalized to Gtf2b. Asterisks indicate a significant effect of the fat source on C57BL/6J or 129S6/SvEvTac mice (n=5).

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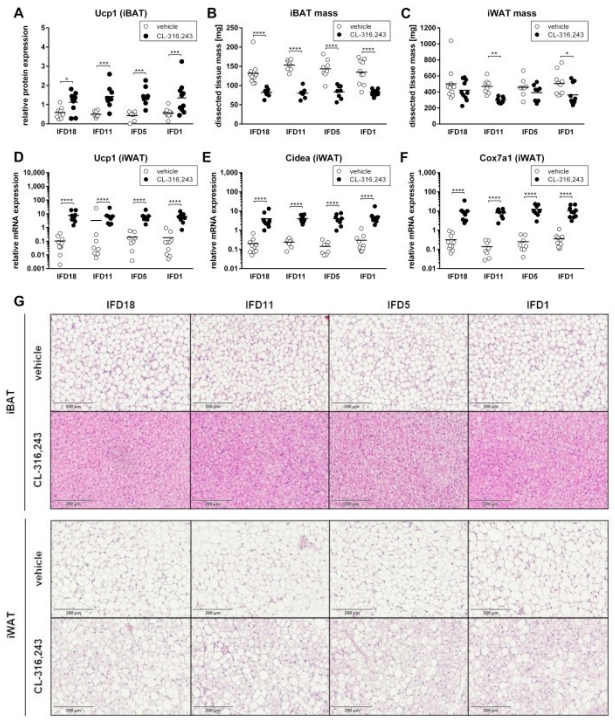

Figure 3: Effect of dietary PUFA-supplementation on the recruitment of thermogenic adipocytes in the presence and absence of β3-agonism. (A) Relative Ucp1 protein levels. Ucp1 was normalized to total protein stain (see Supporting Information Figure S5). (B) Dissected iBAT mass. (C) Dissected iWAT mass. (D-F) Relative gene expression of (D) Ucp1, (E) Cidea and (F) Cox7a1 in iWAT. Gene expression was normalized to Rplp0. (G) Hematoxylin/Eosin-stained iBAT and iWAT sections. Log10 transformed data was used for statistical analysis in B and C in order to meet the assumption of normal distribution. There were no significant effects of the diet among vehicle or CL 316,243 treated mice. Asterisks indicate a significant effect of CL 316,243 versus vehicle treatment (n=8-10).

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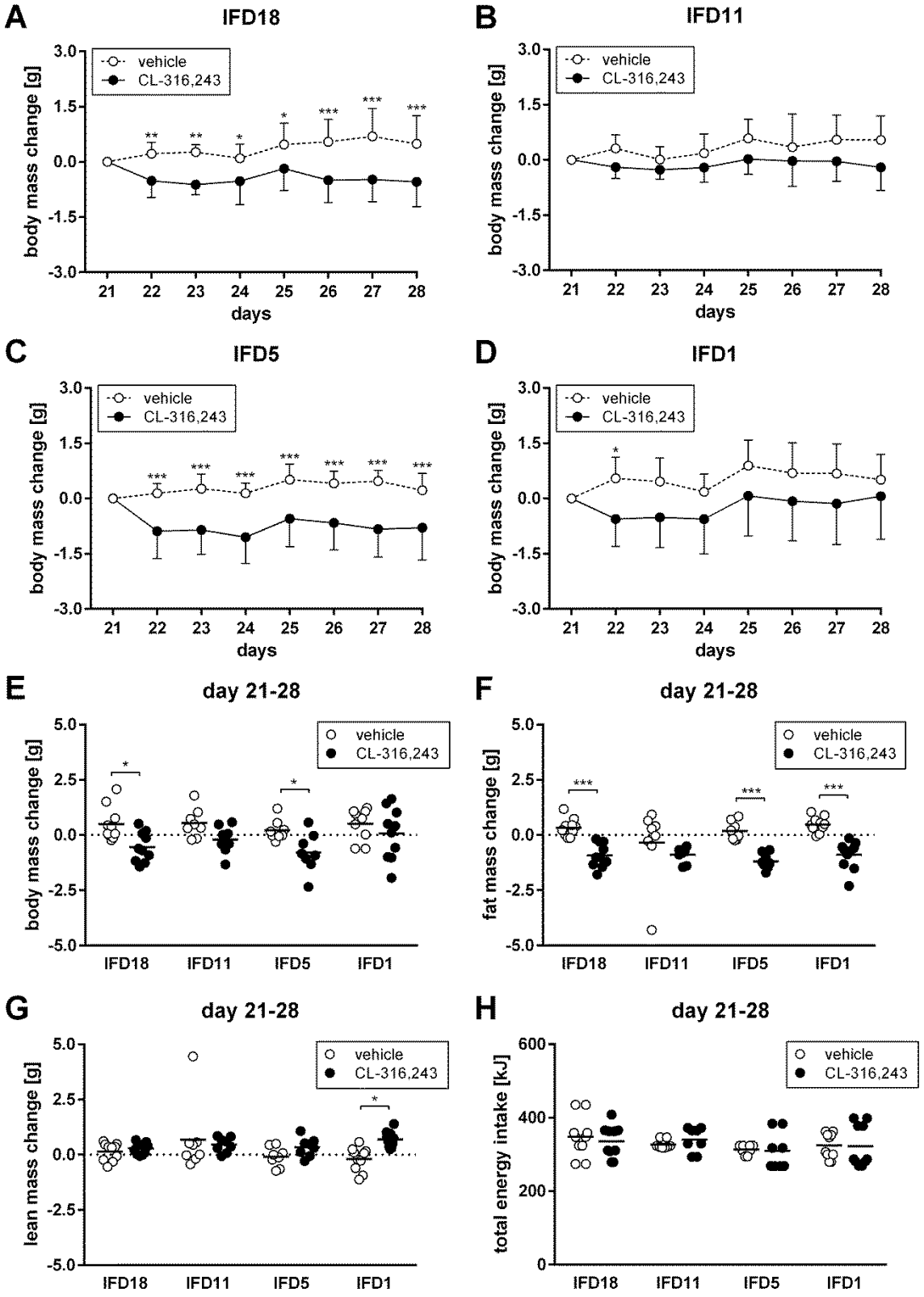

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Figure 4: Effect of dietary PUFA supplementation and β3-agonism on body mass and body composition changes. (A D) Body mass trajectories depicting absolute changes in body mass during vehicle and CL-316,243 treatment of mice fed with (A) IFD18, (B) IFD11, (C) IFD5 and (D) IFD1. (E-G) Total changes in (E) body mass, (F) fat mass and (G) lean mass during 7 days of vehicle and CL-316,243 treatment. (H) Total energy intake during vehicle and CL-316,243 treatment. Asterisks indicate a significant effect of CL 316,243 versus vehicle treatment (n=8-10). There were no significant effects of the diet among vehicle or CL 316,243 treated mice.

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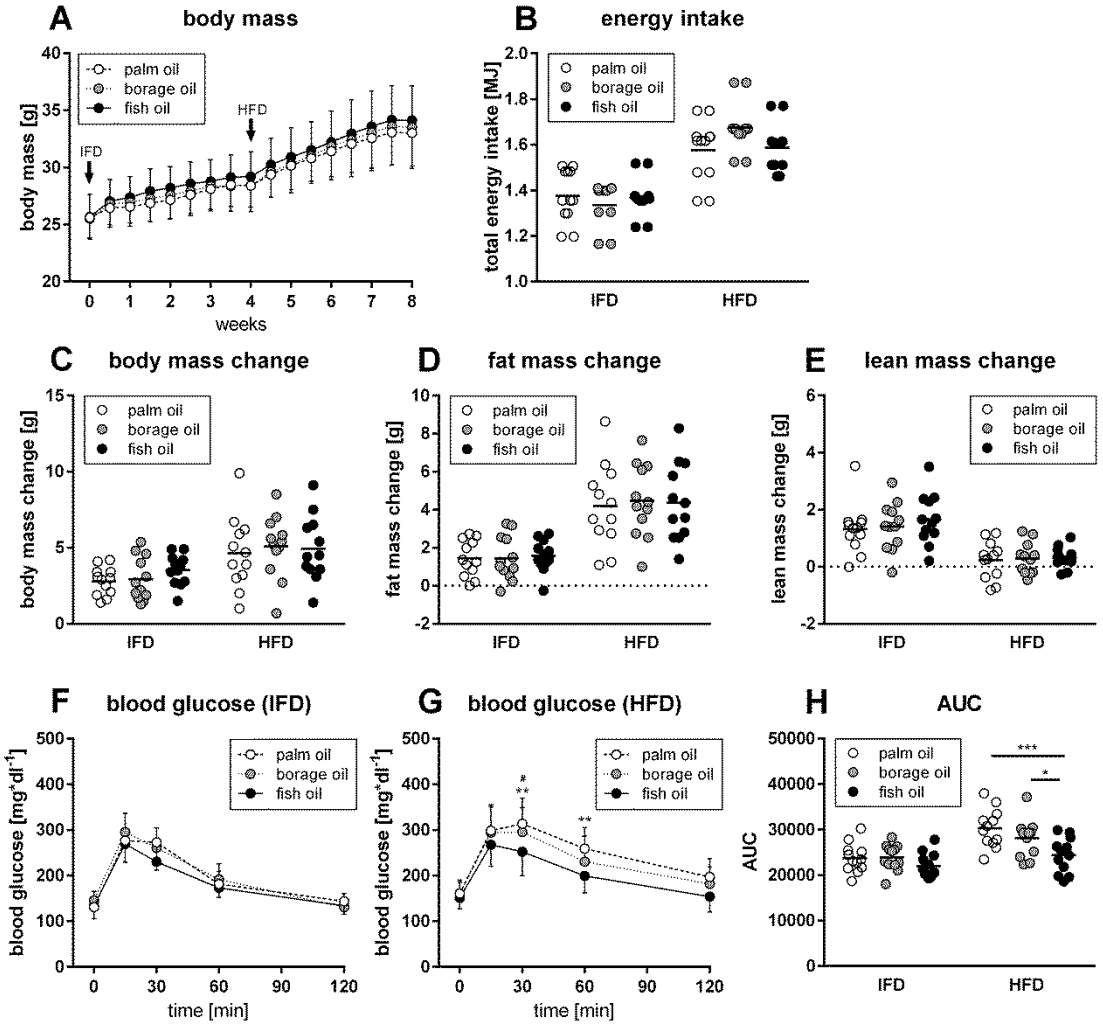

Figure 5: Effect of dietary PUFA supplementation on the development of diet-induced obesity and glucose intolerance in C57BL/6J mice. (A) Body mass trajectories. (B) Total energy intake at the end of IFD and HFD-feeding. (C-E) Changes in (C) body mass, (D) fat mass and (E) lean mass over the course of IFD and HFD-feeding. (F, G) Blood glucose trajectories of oral glucose tolerance tests conducted at the end of (F) IFD-feeding and (G) HFD-feeding. Asterisks indicate a significant effect of fish oil versus palm oil supplementation (n=12). Rhombs indicate a significant effect of fish oil versus borage oil supplementation. There were no significant differences between borage oil and palm oil supplementation. (H) Total area under the curve (AUC) of blood glucose levels. Asterisks indicate a significant effect of the dietary fat source during HFD-feeding (n=12).

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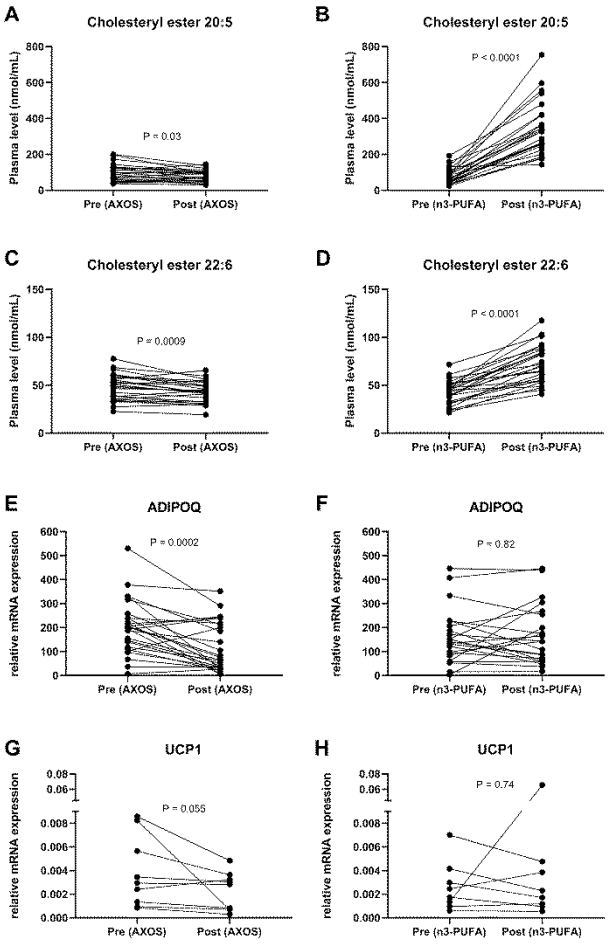

Figure 6: Effect of dietary PUFA supplementation on (A, B) plasma levels of cholesteryl ester 20:5, (C, D) cholesteryl ester 22:6 and gene expression in human WAT of (E,F) adiponectin and (G, H) Ucp1. Overweight and obese subjects supplemented their diet with (A, C, E, G) arabinoxylan oligosaccharides (AXOS) or (B, D, F, H) fish oil (n3-PUFA) over 4 weeks during a randomized crossover trial. Both dietary interventions were separated by a 4-week washout period. Blood plasma and abdominal subcutaneous fat was collected before (pre) and after (post) each dietary intervention. All datasets were analyzed by two tailed, nonparametric Wilcoxon matched-pairs signed rank test with p-values indicated (n=8-26).

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