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ORIGINAL CONTRIBUTION

Dietary supplementation of herring roe and milt enhances hepatic fatty acid catabolism in female mice transgenic for hTNFα

Bodil Bjørndal · Lena Burri · Hege Wergedahl · Asbjørn Svardal · Pavol Bohov · Rolf K. Berge

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Abstract
Purpose The beneficial effects of a seafood-rich diet are highly documented and can be attributed to both *n*-3 polyunsaturated fatty acids and other less studied nutritional components including protein and antioxidants. The aim of the work was to investigate whether an underutilized seafood source, eggs (roe) and sperm (milt) from herring (*Clupea harengus*), can affect lipid metabolism and inflammation in a mouse model transgenic for human tumor necrosis factor alpha (hTNFα).
Methods A high-fat control diet (25% total fats, 20% protein, w/w) or high-fat diets supplemented with herring roe (3.7% fat, 15% protein, w/w), or milt (1.3% fat, 15% protein) were administered to female C57BL/6 hTNFα mice. After 2 weeks, hepatic enzyme activity, gene expression, lipid and fatty acid composition, fatty acid composition of epididymal adipose tissue, and plasma lipid and cytokine levels were determined.
Results Animals fed herring roe and milt displayed an increased hepatic fatty acid β-oxidation and reduced fatty acid synthase activity. However, while plasma TAG was reduced, hepatic TAG and plasma and hepatic cholesterol levels were increased by the herring diets. Both herring diets led to a substantial shift in the *n*-6:*n*-3 ratio in both liver and ovarian white adipose tissue. The herring diets also increased plasma carnitine and reduced the carnitine precursor trimethyllysine. Plasma short-chained acylcarnitine esters were significantly increased, which may reflect an increased β-oxidation of long-chained fatty acids. In addition, the diets tended to reduce the plasma levels of pro-inflammatory cytokines.
Conclusion Herring roe or milt diets enhanced lipid catabolism and influenced the chronic inflammatory state in hTNFα-transgenic mice.

Keywords Herring roe · Herring milt · TNF alpha · High-fat diet · Lipid catabolism and inflammation

Abbreviations

AADAC	Arylacetamide deacetylase
ACACA	Acetyl-CoA carboxylase alpha
ACOX1	Acyl-CoA oxidase 1
ACS	Acyl-CoA synthetase
CPT	Carnitine palmitoyltransferase
CSF2	Colony-stimulating factor 2 (granulocyte-macrophage)
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FADS	Fatty acid desaturase
FASN	Fatty acid synthase
HMGCS	3-hydroxy-3-methylglutaryl-coenzyme a synthase
hTNFα	Human tumor necrosis factor alpha
LDLR	Low-density lipoprotein receptor

B. Bjørndal (✉) · L. Burri · A. Svardal · P. Bohov · R. K. Berge
Institute of Medicine, University of Bergen, 5021 Bergen, Norway
e-mail: bodil.bjorndal@med.uib.no

H. Wergedahl
Faculty of Education, Bergen University College, 5020 Bergen, Norway

R. K. Berge
Department of Heart Disease, Haukeland University Hospital, 5021 Bergen, Norway

PPAR	Peroxisome proliferator-activated receptor
SCD1	Stearoyl-CoA desaturase 1
WAT	White adipose tissue

Introduction

One major feature of metabolic syndrome is obesity especially located in the visceral depot, which can result in dyslipidemia, hypertension, and insulin resistance. These obesity-related disorders, mainly caused by life style habits and intake of energy-rich food [1] are accompanied with chronic inflammation [2]. Accordingly, the pro-inflammatory cytokine tumor necrosis factor (TNF) α is overexpressed in the white adipose tissue (WAT) of obese human subjects as well as in mice models of obesity [3–5]. Moreover, this cytokine has been shown to perturb lipid and cholesterol metabolism. We have previously found downregulated expression of peroxisome proliferator-activated receptor alpha (PPARα) target genes and reduced mitochondrial fatty acid oxidation in the liver of mice transgenic for human TNFα (hTNFα) [6].

The beneficial effect of fish consumption has mainly been attributed to the *n*-3 polyunsaturated fatty acid (PUFA) content of fatty fish [7]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil are thought to bind and activate PPARs [8] and are important anti-inflammatory agents as precursors of eicosanoids and resolvins [9]. However, other nutritional components in fish could also contribute to the overall positive health effect. Several fish protein hydrolysates have been shown to lower plasma lipids in rodent obesity models [10–13]. Fish protein also seems to have reparative properties in rat intestine [14] and immunomodulating effects in BALB/c mice [15]. At the moment, there is a large interest in the action of new, under-utilized bioactive food compounds, for example, derived from fatty fish such as herring (*Clupea harengus*). Roe and milt from fish have a high protein level (70–95%), as well as high levels of *n*-3 PUFAs [16, 17]. The main *n*-3 PUFAs found in milt and roe are EPA and DHA, and they are mainly incorporated in phospholipids [17, 18].

While the use of salted roe (egg) from different fish species is relatively widespread, milt (sperm) is traditionally used for human consumption only in a few countries. Due to its high nutritional value, herring milt has recently been introduced as a food additive for malnourished children in developing countries. In Norway, of the approximately 17,000 tons herring roe produced every year, only a few tons go directly for human consumption. The rest is further processed into oils and proteins together with other herring byproducts. Thus, there is a potential to expand the commercially viable use of both these products.

The effect of herring milt on lipid metabolism has not been studied previously; however, a combination of herring roe protein and lipids was found to reduce plasma lipid levels and abdominal fat pad weight in mice on high-fat diets [19]. A few studies have been performed on a Japanese salted herring roe, called kazunoko, which is a caviar-type rich in cholesterol, EPA, and DHA. Kazunoko was shown to reduce plasma lipid levels in male Crlj:CD-1 (ICR) mice [20]. It also reduced plasma glucose in this model, most likely due to a stimulated adiponectin release.

The purpose of the present study was to determine whether herring roe and milt had the potential to modulate lipid metabolism and inflammation in a mouse model of chronic low-grade inflammation due to hTNFα expression and a high-fat diet.

Materials and methods

Animals and diets

In this study, female transgenic mice expressing the hTNFα gene was used (Taconic, Germantown, USA). This mouse line has been generated in the strain C57Bl/6 and expresses low levels of hTNFα [21]. The experiments were performed in accordance with, and under the approval of, the Norwegian State Board for Biological Experiments, the Guide for the Care and Use of Laboratory Animals, and the Guidelines of the Animal Welfare Act. Mice between 6 and 8 weeks of age were divided into three experimental groups of five animals each with comparable mean body weight and were housed in cages with constant temperature (22 ± 2 °C) and humidity (55 ± 5%). They were exposed to a 12-h light–dark cycle and had unrestricted access to tap water and food. The mice were acclimatized to these conditions before the start of the experiment and were given standard chow during the acclimatizing period.

Mice were fed *ad libitum* for 2 weeks on complete diets containing 20% (w/w) protein from bovine milk casein (Tine, Tolga, Norway), or a mixed protein source from casein (5%, w/w) and herring roe (egg), or herring milt (sperm) (15%, w/w), as described in Table 1. Herring supplements were spray-dried (provided by Dr. Alfred Halstensen). The amino acid compositions of the spray-dried diet supplements are given in Table 2. All diets contained 25% (w/w) fat consisting of lard (Ten Kate Vetten BV, Musselkanaal, Netherlands), 2% soy oil (Dyets Inc., Bethlehem, PA, USA), and fat contribution from the herring supplements (Table 1). The fatty acid composition of the diets is given in Table 3, and the distribution of lipid classes in spray-dried herring roe and milt is given in Table 4. The

Table 1 Composition of the experimental diets

Ingredients	*C* (g/kg of diet)	*HR* (g/kg of diet)	*HM* (g/kg of diet)
Lard	230	193.2	216.7
Soy oil	20	20	20
Casein[a]	238.7	59.7	59.7
Herring roe[b]	–	207.5	–
Herring milt[c]	–	–	175.2
Fish hydrolysate	–	–	–
Lipid content of HR and HM	–	(36.8)	(13.3)
Cornstarch	179	187	196
Dyetrose	132	132	132
Sucrose	100	100	100
Fiber	50	50	50
AIN-93G mineral mix	35	35	35
AIN-93 vitamin mix	10	10	10
L-cysteine	3	3	3
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014

C control diet, *HR* herring roe, *HM* herring milt

The diets were isoenergetic and isonitrogenous and contained 20 g of protein per 100 g of diet

[a] Casein consisted of 83.8% (w/w) protein and 0.2% fat

[b] Herring roe consisted of 72.3% protein and 17.7% fat

[c] Herring milt consisted of 85.6% protein and 7.6% fat

Table 2 AA composition of *herring roe* (HR) and *milt* (HM)

Amino acid	*HR* (g/6.25 g N)	*HM* (g/6.25 g N)
Asp	7.9	3.3
Glu	12.3	4.7
Hpr	0.3	0.0
Ser	6.0	2.7
Gly	4.1	5.2
His	2.5	1.0
Arg	5.3	15.5
Thr	5.7	2.5
Ala	9.0	3.0
Pro	5.7	2.8
Tyr	3.7	1.4
Val	6.7	2.7
Met	2.7	1.4
Ile	5.9	1.9
Leu	10.6	3.6
Phe	3.4	1.8
Lys	7.3	3.1
Cys	–	1.0
Trp	–	0.1

Table 3 Fatty acid composition of the experimental diets

Fatty acid	Diet groups		
	C (%, w/w)	*HR* (%, w/w)	*HM* (%, w/w)
$\sum$ SFAs	44.0	41.8	42.6
C16:0	25.4	25.0	24.7
C18:0	16.0	14.0	15.4
$\sum$ MUFAs	38.8	36.6	37.9
C16:1*n*-7	1.62	1.97	1.59
C18:1*n*-7	2.29	2.64	2.53
C18:1*n*-9	33.8	30.8	32.7
$\sum$ *n*-6 PUFAs	15.6	14.5	15.2
C18:2*n*-6	14.9	13.5	14.4
C20:3*n*-6	0.08	0.07	0.07
C20:4*n*-6	0.18	0.33	0.24
$\sum$ *n*-3 PUFAs	1.51	7.04	4.22
C18:3*n*-3	1.34	1.28	1.31
C20:5*n*-3	0.02	2.05	0.60
C22:5*n*-3	0.08	0.26	0.26
C22:6*n*-3	0.05	3.26	1.97
$\sum$ *n*-6:$\sum$ *n-3* ratio	10.3	2.05	3.59

C control diet, *HR* herring roe, *HM* herring milt *SFAs* saturated fatty acids *MUFAs* monounsaturated fatty acids *PUFAs* polyunsaturated fatty acids

Table 4 Lipid class content in *herring roe* (HR) and *milt* (HM)

Lipid classes	*HR* (%, w/w)	*HM* (%, w/w)
Triglycerides (TAG)	18.4	4.2
Diglycerides (DAG)	<0.5	<0.5
Monoglycerides (MAG)	<1	<1
NEFA	4.3	18.1
Cholesterol	8.8	17.5
Cholesterol esters (CE)	0.3	0.3
Phosphatidyl choline (PC)	56.3	38.2
Phosphatidyl etanolamine (PE)	8.9	19.4

other constituents of the diets were cornstarch, dyetrose, sucrose, fiber, AIN-93G mineral mix, AIN-93 vitamin mix, L-Cysteine, Choline bitartrate (all from Dyets Inc.), and tert-Butyl-hydroquinone (Sigma–Aldrich).

The mice were anaesthetized under fasting conditions by inhalation of 2% isoflurane (Schering-Plough, Kent, UK) after 2 weeks of diet treatment. Blood was collected by aortic puncture with 7.5% EDTA and immediately chilled on ice. Plasma was prepared and stored at −80 °C prior to analysis. Parts of the liver was chilled on ice and used for β-oxidation analysis, and the rest was freeze-clamped and stored at −80 °C until further analysis. Epididymal WAT was dissected, freeze-clamped, and stored at −80 °C.

Plasma and hepatic lipids

Lipid classes in spray-dried herring roe and milt were determined with LC/CAD according to Xiao et al. [22]. Liver lipids were extracted according to the method proposed by Bligh and Dyer [23], evaporated under nitrogen, and redissolved in isopropanol before analysis. Lipids were then measured enzymatically on a Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany) using the triacylglycerol (GPO-PAP) and cholesterol kit (CHOD-PAP) from Roche Diagnostics, and the phospholipid kit from bioMérieux SA (Marcy l'Etoile, France). Plasma lipids were measured directly on the Hitachi 917 system using the above mentioned kits, and in addition the HDL-cholesterol kit and LDL-cholesterol kit from Roche, and the non-esterified fatty acid (NEFA) FS kit and free cholesterol FS kit from Diasys (Diagnostic Systems GMbH, Holzheim, Germany).

Hepatic and WAT fatty acid and plasma carnitine compositions

Total hepatic and epididymal WAT fatty acid composition was analyzed as described previously [6]. Free carnitine, short-, medium-, and long-chain acylcarnitines, and the precursors for carnitine, trimethyllysine and butyrobetaine, were analyzed in plasma using MS/MS as described previously [24] with some modifications of the HPLC conditions: The LC system was an Agilent (Waldbronn, Germany) 1200 Series with binary pump, variable volume injector, and a thermostated autosampler. HPLC separation was conducted at 30 °C using a gradient solvent mixture. Mobile phase A was made of 10 mM ammonium acetate and 12 mM HFBA in water, and mobile phase B was made of 10 mM ammonium acetate and 12 mM HFBA in methanol. The gradient was B 0.1 min 20%, flow 0.2 mL/min; B 4 min 20–90%, flow 0.2 mL/min; B 14 min 90%, flow 0.2 mL/min; B 10 min 2%, flow 0.6 mL/min; B 0.1 min 20%, flow 0.2 mL/min. A Phenomenex Luna C8 column (5 µm, 150 × 2 mm) equipped with a Phenomenex C18 pre-column, (4.0 × 2.0 mm) was used. Two microliters of the sample were injected.

Hepatic enzyme activities

The livers were homogenized and fractionated as described earlier [25]. Palmitoyl-CoA oxidation was measured in a mitochondria-enriched extract from liver as acid-soluble products [26]. The activities of carnitine palmitoyltransferase (CPT) 1 and CPT2 and acyl-CoA synthetase (ACS) [27] were measured in the mitochondrial fraction. The activities of fatty acid synthase (FAS) and acyl-CoA oxidase (ACOX) were measured in the post-nuclear fraction as described by Skorve et al. [28].

Gene expression analysis

Total cellular RNA was purified from 20 to 30 mg of frozen tissue samples from liver using a TissueLyser II with 5-mm stainless steel beads, and the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified spectrophotometrically by NanoDrop 1000 (NanoDrop Technologies, Boston, MA USA), and the quality was evaluated by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). For each sample, 1 mg of total RNA was reversely transcribed in 100 µL reactions using Applied Biosystems' Taq man Reverse Transcription Reagents with RNase inhibitor according to the manufacturer's description (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with Sarstedt 384-well multiply-PCR plates (Sarstedt Inc., Newton, NC, USA) on the following genes, using probes and primers from Applied Biosystems: acetyl-CoA carboxylase alpha (*Acaca*, Mm01304277_m1), *Acox1* (Mm00443579), CD36 antigen (*CD36* (*Fat*), Mm00432403), *Cpt1a* (Mm00550438), *Cpt2* (Mm00487202), cytochrome P450, family 7, subfamily A, polypeptide 1 (*Cyp7a1*, Mm00484152), hydroxyl-CoA dehydrogenase (trifunctional protein) alpha (*Hadha*, Mm00805228_m1), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*Hmgc1,* Mm00524111), *Hmgc2* (Mm00550050), low-density lipoprotein receptor (*Ldlr,* Mm00440169), and stearoyl-CoA desaturase 1 (*Scd1*, Mm00772290_m1). Probes and primers from Thermo Fisher Scientific Inc. (Waltham, MA, USA) were used on the genes: arylacetamide deacetylase (*Aadac*, Gene ID 67758), fatty acid desaturase 1 (*Fads1*, Gene ID 76267), *Fads2* (Gene ID 56473). Three different reference genes were included: 18*s* [Kit-FAM-TAMRA (Reference RT-CKFT-18s)] from Eurogentec, Belgium, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1) from Applied Biosystems, and ribosomal protein, large, P0 (*Rplp0*, Gene ID 11837) from Thermo Fisher Scientific. To evaluate the reference genes, we used NormFinder, and data normalized to *Rplp0* are presented.

Cytokine and adiponectin measurements

The levels of interleukin (IL)-1β, IL-2, IL-5, and granulocyte-macrophage colony-stimulating factor (CSF2) in plasma were assessed in a 96-well plate assay using custom-made four-plex kits (Millipore Corp., St. Charles, IL, USA). Plasma adiponectin was measured using a single-plex kit (Millipore). The analysis was performed on undiluted plasma samples, in an overnight protocol according to the manufacturer's recommendations, and data were collected using the Bio-Plex 200 system (BioRad, Hercules, CA, USA).

Statistical analysis

Data sets were analyzed using Prism Software (Graph-Pad Software, San Diego, CA) to determine statistical significance. The results are shown as means of five animals per group ($n = 5$) with their standard deviations. Unpaired t test was performed to evaluate statistical differences between groups, or Mann–Whitney test when values were not normally distributed. P values ≤ 0.05 were considered significant.

Table 5 Body weight, liver index, and feed intake of hTNFα-mice fed *control* (C), *herring roe* (HR), or *herring milt* (HM) diets for 2 weeks

Parameters	Diet groups		
	C	*HR*	*HM*
Start weight (g)	19.2 ± 1.1	20.2 ± 1.3	20.4 ± 0.6
End weight (g)	20.6 ± 1.5	23.8 ± 2.4*	23.2 ± 2.5
Weight gain (g)	1.4 ± 0.6	3.6 ± 1.7*	2.8 ± 1.3*
Liver index (% of body weight)	4.3 ± 0.3	4.6 ± 0.3	4.6 ± 0.3
Feed intake (g mouse/day)	2.44	2.82	2.59
Feed efficiency (weight gain/feed intake)	0.04 ± 0.02	0.09 ± 0.04*	0.08 ± 0.04

Data are means ± SD ($n = 5$). Values statistically different from control by t test are indicated (* $P < 0.05$)

Results

Plasma and hepatic lipids

The herring diets gave a small but significant increase in weight gain and displayed an increased feed efficiency compared to control, although only significant for herring roe (Table 5). The herring roe diet increased the plasma total cholesterol, both the LDL- and HDL-cholesterol levels, significantly compared to the control diet (Fig. 1a). In contrast, herring milt had no effect on total and HDL-plasma cholesterol and only gave a small, but significant, increase in LDL-cholesterol. As a result, the chol-LDL/chol-HDL ratio was significantly increased by both diets (data not shown). Interestingly, the plasma TAG level was significantly lowered by herring milt ($P = 0.037$) treatment compared to control in transgenic TNFα mice (Fig. 1b). The plasma phospholipids were not affected (Fig. 1c), nor were the plasma NEFA and glucose levels (data not shown).

Both hepatic cholesterol and TAG were increased after herring roe and milt feeding compared to control (Fig. 1d, e). While herring roe had the major cholesterol elevating effect in plasma, the herring milt diet increased hepatic cholesterol significantly more than the herring roe diet (t test, $P = 0.003$). In addition, the total hepatic phospholipids (Fig. 1f) increased after herring roe and milt feeding compared to control.

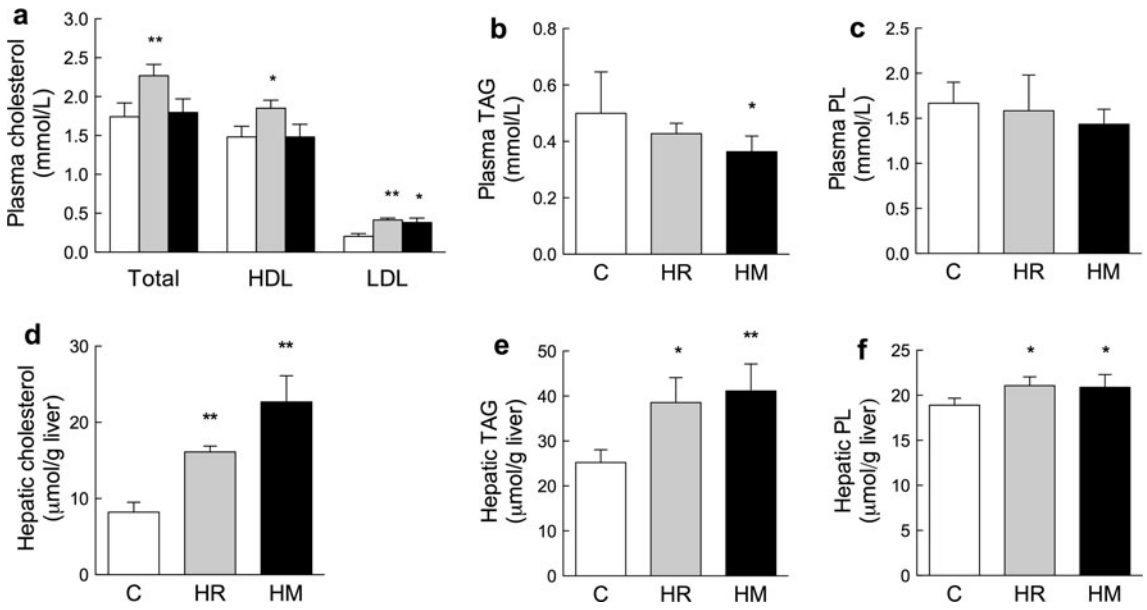

Fig. 1 The effect of 2 weeks herring roe and milt diets on plasma levels of HDL-cholesterol **a**, TAG **b**, phospholipids **c**, and hepatic cholesterol **d**, TAG **e** and phospholipids **f** in hTNFα transgenic mice. Data are means ± SD ($n = 5$) of control (*white bars*), herring roe (*gray bars*), and herring milt (*black bars*). Values significantly different from control are indicted (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). *C* control, *HM* herring milt, *HR* herring roe, *PL* phospholipid, *TAG* triacylglycerol

Fatty acid composition in liver and epididymal WAT

Dietary supplementation with herring roe and milt changed the fatty acid composition in the liver as well as in the epididymal WAT compared to the control diet (Table 6). It was of interest to note that the weight % of SFAs was significantly reduced by the herring diets in the liver, but increased in WAT (Table 6). In the latter tissue, this was due to increased C16:0 and especially C18:0. Conversely, hepatic levels of C16:0 and C18:0 were decreased.

The relative amount of MUFAs was directed in an opposite manner in the two tissues (Table 6). Marked increased levels of in particular C18:1*n*-9 was observed in the liver, whereas this fatty acid was decreased in the WAT by herring roe and milt (Table 6). This made a significant increase in the C18:1*n*-9/C18:0 desaturation index in liver and a concurrent decrease in ovarian WAT with herring diets compared to control (Table 6). However, a hepatic upregulation of the desaturase SCD1 could not be confirmed at the mRNA level (Table 7).

The level of *n*-3 PUFAs was increased in both liver and WAT by herring milt and roe compared to control mice (Table 6). This was due to increased relative amounts of in particular C18:3*n*-3, C20:5*n*-3, C22:5*n*-3, and C22:6*n*-3. The *n*-6 PUFAs were decreased by the dietary supplementation mainly in the liver, and the effects were related

Table 6 Fatty acid compositions in liver and epididymal WAT from female TNFα transgenic mice given a high-fat *control diet* (C) or diets supplemented with *herring roe* (HR) or *milt* (HM) for 2 weeks

Fatty acids	Liver			WAT		
	C (%, w/w)	*HR* (%, w/w)	*HM* (%, w/w)	*C* (%, w/w)	*HR* (%, w/w)	*HM* (%, w/w)
∑ SFAs	34.6 ± 0.7	32.2 ± 2.1	30.8 ± 0.7***	32.4 ± 1.1	35.8 ± 1.0***	34.9 ± 1.3*
C14:0	0.27 ± 0.02	0.30 ± 0.05	0.29 ± 0.06	1.57 ± 0.10	1.81 ± 0.05**	1.70 ± 0.08*
C16:0	21.4 ± 0.4	20.7 ± 1.3	20.1 ± 0.4***	25.3 ± 0.8	27.3 ± 0.7**	26.1 ± 0.6
C18:0	12.0 ± 0.6	10.3 ± 0.9**	9.51 ± 0.92***	4.61 ± 0.45	5.64 ± 0.25**	6.20 ± 0.75**
∑ MUFAs	25.4 ± 0.9	30.0 ± 2.7**	30.5 ± 3.2**	48.6 ± 1.2	44.4 ± 0.8***	46.2 ± 2.0*
C16:1*n*-7	1.03 ± 0.13	1.30 ± 0.16*	1.16 ± 0.30	4.74 ± 0.34	4.57 ± 0.24	4.35 ± 0.75
C16:1*n*-9	0.40 ± 0.04	0.45 ± 0.08	0.47 ± 0.10	0.45 ± 0.02	0.36 ± 0.02***	0.33 ± 0.01***
C18:1*n*-7	1.60 ± 0.08	1.34 ± 0.13**	1.23 ± 0.15***	2.22 ± 0.08	1.98 ± 0.04***	2.06 ± 0.03**
C18:1*n*-9	21.8 ± 0.75	26.3 ± 2.4**	27.1 ± 2.7**	40.4 ± 1.4	36.6 ± 0.7***	38.6 ± 1.4
∑ *n*-6 PUFAs	31.1 ± 0.6	22.4 ± 1.0***	22.7 ± 1.4***	17.6 ± 1.4	16.4 ± 1.1	15.7 ± 1.4
C18:2*n*-6	16.0 ± 0.5	15.9 ± 1.0	16.9 ± 2.0	16.7 ± 1.4	15.7 ± 1.1	15.1 ± 1.4
C18:3*n*-6	0.32 ± 0.03	0.18 ± 0.02***	0.18 ± 0.01***	<0.1	<0.1	<0.1
C20:2*n*-6	0.24 ± 0.01	0.17 ± 0.00***	0.16 ± 0.01***	0.24 ± 0.02	0.22 ± 0.01	0.24 ± 0.01
C20:3*n*-6	0.90 ± 0.06	0.99 ± 0.11	0.69 ± 0.09***	0.15 ± 0.01	0.12 ± 0.00**	0.10 ± 0.01***
C20:4*n*-6	13.0 ± 0.8	5.06 ± 0.55***	4.67 ± 0.64***	0.34 ± 0.04	0.21 ± 0.01**	0.20 ± 0.01**
C22:4*n*-6	0.34 ± 0.01	<0.1***	<0.1***	<0.1	<0.1	<0.1
C22:5*n*-6	0.28 ± 0.03	<0.1***	<0.1***	<0.1	<0.1	<0.1
∑ *n*-3 PUFAs	8.73 ± 0.65	15.2 ± 2.2**	15.9 ± 1.8***	1.28 ± 0.10	3.39 ± 0.25***	3.02 ± 0.28***
C18:3*n*-3	0.42 ± 0.03	0.59 ± 0.03***	0.70 ± 0.05***	0.89 ± 0.06	1.12 ± 0.05***	1.02 ± 0.07*
C20:5*n*-3 (EPA)	0.29 ± 0.03	2.53 ± 0.36***	2.89 ± 0.25**	0.03 ± 0.01	0.33 ± 0.04***	0.29 ± 0.04**
C22:5*n*-3	0.29 ± 0.02	0.69 ± 0.07**	0.74 ± 0.05***	0.07 ± 0.01	0.17 ± 0.01***	0.17 ± 0.01***
C22:6*n*-3 (DHA)	7.60 ± 0.65	11.2 ± 1.7**	11.3 ± 1.5***	0.24 ± 0.03	1.66 ± 0.17**	1.47 ± 0.17**
Other FAs	2.19 ± 0.19	1.94 ± 0.24	1.80 ± 0.24	1.96 ± 0.22	2.54 ± 0.15	2.00 ± 0.21
∑ *n*-6:∑ *n*-3 ratio	3.57 ± 0.21	1.49 ± 0.20**	1.44 ± 0.13***	13.8 ± 1.3	4.86 ± 0.62***	5.23 ± 0.41***
Δ5 (*n*-6) desat. index[a]	14.4 ± 1.4	5.16 ± 0.59***	6.86 ± 0.97***	2.32 ± 0.30	1.77 ± 0.07**	1.92 ± 0.08*
Δ6 (*n*-6) desat. index[b]	22.0 ± 3.2	11.7 ± 2.1***	10.9 ± 0.7***	4.00 ± 0.61	3.41 ± 0.37	3.03 ± 0.15**
Δ9 desat. index[c]	1.81 ± 0.12	2.59 ± 0.43**	2.89 ± 0.56**	8.82 ± 0.76	6.49 ± 0.33***	6.30 ± 0.84***

Data are means ± SD ($n = 5$). Values significantly different from control are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

[a] Δ5 (*n*-6) desaturase index = 20:4*n*-6/20:3*n*-6

[b] Δ6 (*n*-6) desaturase index = 18:3*n*-6/18:2*n*-6, $\times 10^{-3}$

[c] Δ9 desaturase index = 18:1*n*-9/18:0

Table 7 Hepatic gene expression in TNFα mice after 2 weeks of *control* (C), *herring roe* (HR), or *herring milt* (HM) diets

Gene	Function	Diet groups			*P* values	
		C	*HR*	*HM*	*C* versus *HR*	*C* versus *HM*
Cpt1a	β-Oxidation	1.00 ± 0.18	1.37 ± 0.18	0.93 ± 0.07	NS	NS
Cpt2	β-Oxidation	1.00 ± 0.23	1.82 ± 0.31	1.47 ± 0.21	0.001	0.009
Acox1	β-Oxidation	1.00 ± 0.17	1.77 ± 0.32	1.34 ± 0.19	0.002	0.017
Hadha	β-Oxidation	1.00 ± 0.11	1.42 ± 0.25	1.22 ± 0.06	0.008	0.004
Aadac	TAG lipase activity	1.00 ± 0.25	1.06 ± 0.16	1.10 ± 0.16	NS	NS
Hmgcs2	Keton body formation	1.00 ± 0.14	1.56 ± 0.17	1.41 ± 0.17	<0.001	0.003
Hmgcs1	Cholesterol synthesis	1.00 ± 0.27	0.34 ± 0.07	0.29 ± 0.03	<0.001	<0.001
Cyp7a1	Bile formation	1.00 ± 0.14	1.12 ± 0.48	1.38 ± 0.34	NS	0.049
Acaca	Fatty acid synthesis	1.00 ± 0.10	0.75 ± 0.18	0.90 ± 0.20	0.027	NS
Cd36	Fatty acid import	1.00 ± 0.21	2.74 ± 1.39	1.82 ± 0.62	0.024	0.023
Ldlr	Cholesterol/lipid import	1.00 ± 0.12	0.65 ± 0.17	0.63 ± 0.19	0.016	0.006
Scd1	Δ9 Desaturase activity	1.00 ± 0.26	1.16 ± 0.42	1.04 ± 0.31	NS	NS
Fads1	Δ5 Desaturase activity	1.00 ± 0.18	0.48 ± 0.13	0.41 ± 0.10	<0.001	<0.001
Fads2	Δ6 Desaturase activity	1.00 ± 0.17	0.55 ± 0.11	0.41 ± 0.08	0.008	0.008

Aadac arylacetamide deacetylase, *Acaca* acetyl-coA-carboxylase, alpha, *Acox1* acyl-CoA oxidase 1, palmitoyl, *Cd36* CD36 molecule/fatty acid translocase, *Cpt1a* carnitine palmitoyltransferase 1A, *Cpt2* carnitine palmitoyltransferase 2, *Cyp7a1* cytochrome P450, family 7, subfamily A, polypeptide 1, *Fads1* Δ5 desaturase/fatty acid desaturase 1, *Fads2* Δ6 desaturase/fatty acid desaturase 2, *Hadha* hydroxyacyl-CoA dehydrogenase (trifunctional enzyme), alpha subunit, *Hmgcs1* 3-hydroxy-3-methylglutaryl-CoA synthase 1, *Hmgcs2* HMG-CoA synthase 2, *Ldlr* low-density lipoprotein receptor, *NS* not significant, *Scd1* stearoyl-coenzyme A desaturase 1

Data are means ± SD ($n = 5$) normalized to *Rplp0* and relative to control. Results were analyzed by unpaired *t* test, with significant *P* values shown ($P < 0.05$)

to decreased levels of most *n*-6 fatty acids except C18:2*n*-6 (Table 6). Altogether, the herring diets resulted in a shift in the ratio of *n*-6 to *n*-3 PUFAs from 3.6:1 to 1.5:1 in liver, and from approximately 14:1 to 5:1 in WAT (Table 6).

Desaturation and elongation are involved in the biosynthesis of *n*-3 and *n*-6 fatty acids. Along with the increased dietary intake of *n*-3 PUFAs in the herring roe and milt groups, the hepatic mRNA levels of the Δ5 desaturase FADS1 and the Δ6 desaturase FADS2 were significantly reduced compared to control (Table 7). The ratio between 20:4*n*-6 and 20:3*n*-6 (Δ5 desaturase index), and 18:3*n*-6 and 18:2*n*-6 (Δ6 desaturase index), also indicated a decreased activity of the Δ5 and Δ6 desaturases in both epididymal WAT and liver (Table 6).

Hepatic fatty acid oxidation and ketogenesis

The β-oxidation of C14 palmitoyl-CoA measured as soluble products in the presence or absence of malonyl-CoA was insignificantly increased in the transgenic TNFα mice after treatment with herring roe and milt (Fig. 2a). However, the malonyl-CoA sensitivity was significantly reduced by both herring diets (Fig. 2b).

Activation of fatty acids to their CoA-esters, catalyzed by ACS, was stimulated by herring roe and milt (Fig. 3a). The transgenic mice exhibited increased, although insignificantly, activity of CPT1 with both herring diets (Fig. 3b), while the mRNA level of CPT1 was unchanged (Table 7). The activities of CPT2 (Fig. 3c) and ACOX1 (Fig. 3d) were significantly increased, and an upregulation of these enzymes could be confirmed at the mRNA level (Table 7). In addition, the hepatic mRNA level of the alpha subunit of the trifunctional enzyme, HADHA, was increased after herring roe administration compared to control (Table 7). CD36, which is involved in transport of long-chained fatty acids, was significantly increased at the mRNA level after herring roe treatment (Table 7). Gene expression of AADAC, an enzyme that is believed to display TAG lipase activity in the liver, was not affected by the herring diets. The mRNA level of HMG-CoA synthase 2 (HMGCS2) was increased in the herring roe group (Table 7). While HMGCS2 is involved in keton body synthesis in mitochondria, the cytosolic HMGCS1 is involved in cholesterol synthesis, and gene expression of *Hmgsc1* was significantly reduced by the herring diets. In addition, both herring roe and milt reduced gene expression of *Ldlr* compared to control. Interestingly, only herring roe led to a significant increase in the mRNA expression of CYP7A1, the rate-limiting enzyme controlling the synthesis of bile acids from cholesterol (Table 7). Strikingly, the FASN activity was strongly inhibited by both herring diets (Fig. 3e), and herring roe also led to a lower

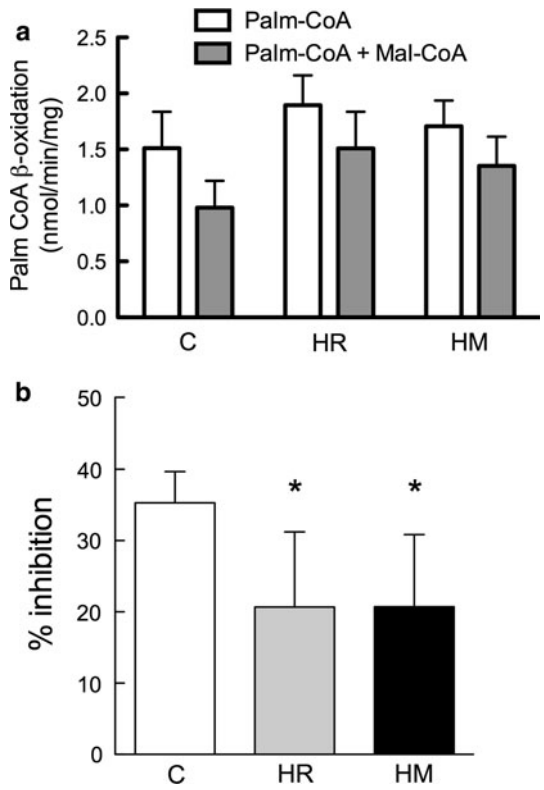

Fig. 2 Palmitoyl-CoA β-oxidation in liver mitochondria of herring roe and milt-treated mice **a**, and the sensitivity for inhibition of oxidation of palmitoyl-CoA with malonyl-CoA given in % inhibition **b**. Oxidation of palmitoyl-CoA was measured in purified mitochondria as acid-soluble products. Data are given as means ± SD ($n = 5$), and values significantly different from control are indicted (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). *C* control, *HM* herring milt, *HR* herring roe, *Mal-CoA* malonyl-CoA, *Palm-CoA* palmitoyl-CoA

expression level of ACACA mRNA (Table 7), suggesting reduced lipogenesis.

Plasma acyl-carnitines

Plasma acyl-carnitines may give an indication on the metabolic status of an animal, since they are intermediary products of β-oxidation, amino acid metabolism, and glycolysis. Both the herring roe and milt diet significantly increased the free carnitine plasma level (Fig. 4a). Correspondingly, the carnitine precursor trimethyllysine was significantly reduced, while its direct precursor γ-butyrobetaine increased with the herring roe and milt diet (Fig. 4b, c). Plasma acetylcarnitine was significantly increased in animals given a herring roe or milt diet compared to control (Fig. 4d). Interestingly, the odd-chained propionylcarnitine was also significantly increased in plasma by both herring diets (Fig. 4e). In contrast, the plasma levels of palmitoylcarnitine (C16) and octanoylcarnitine (C8) were not affected in this study (Fig. 4f, g).

Plasma cytokines

To investigate whether the herring diets were able to influence the inflammatory state of the hTNFα-mice, plasma cytokine levels of IL-1β, IL-2, IL-5, and CSF2 were measured (Fig. 5a). Interestingly, herring roe and milt tended to reduce all the cytokines measured, and herring roe reduced IL-5 significantly compared to control. In addition, in particular herring roe tended to increase the plasma adiponectin level compared to control (Fig. 5b).

Discussion

The metabolic syndrome is a collection of metabolic abnormalities including not only hypertension, obesity, insulin resistance, but also high and low levels of plasma TAG and HDL-cholesterol, respectively. These obesity-related metabolic disorders are closely associated with chronic inflammation and moreover, diets enriched in SFAs have been associated with increased risk for atherosclerosis [29]. Inflammation induces a variety of metabolic changes and affects expression of key proteins involved in lipid metabolism. Whereas SFAs have been shown to promote the metabolic syndrome by activating the Toll-like receptor [30], TNFα is an inflammatory factor that downregulates PPARα and PPARα target genes in rat liver [6, 31]. The purpose for this study was to investigate whether herring roe and milt were able to counteract TNFα-induced metabolic aberrations in female mice fed a high-fat diet.

In the present study, we showed that herring roe and milt lowered plasma TAG, albeit only significantly by herring milt (Fig. 1). Moreover, herring roe and milt stimulated the hepatic mitochondrial CPT2 activity, peroxisomal fatty acid oxidation, and ACS activities (Fig. 3), accompanied by a reduced mitochondrial β-oxidation sensitivity to malonyl-CoA and increased plasma acetylcarnitine levels (Fig. 2). There is a correlation between the activities of CPT2 and HMG-CoA synthase 2 [32], as increased β-oxidation will produce acetyl-CoA, which can be used for keton body formation. In agreement with this, herring roe and milt significantly increased expression of HMGCS2 mRNA. Despite the increase in β-oxidation, the hepatic TAG level, already reported to be elevated in the transgenic mice [6], was further elevated by the herring diets. The small increase observed in feed intake in herring mice versus control mice was not likely to explain this rise in heptic TAG. The exact reasons why hepatic TAG is increased in hTNFα-mice submitted to standard feeding are not yet elucidated at the molecular level and will require further detailed studies. Impaired mobilization of TAG stores is probably not the reason for hepatic TAG

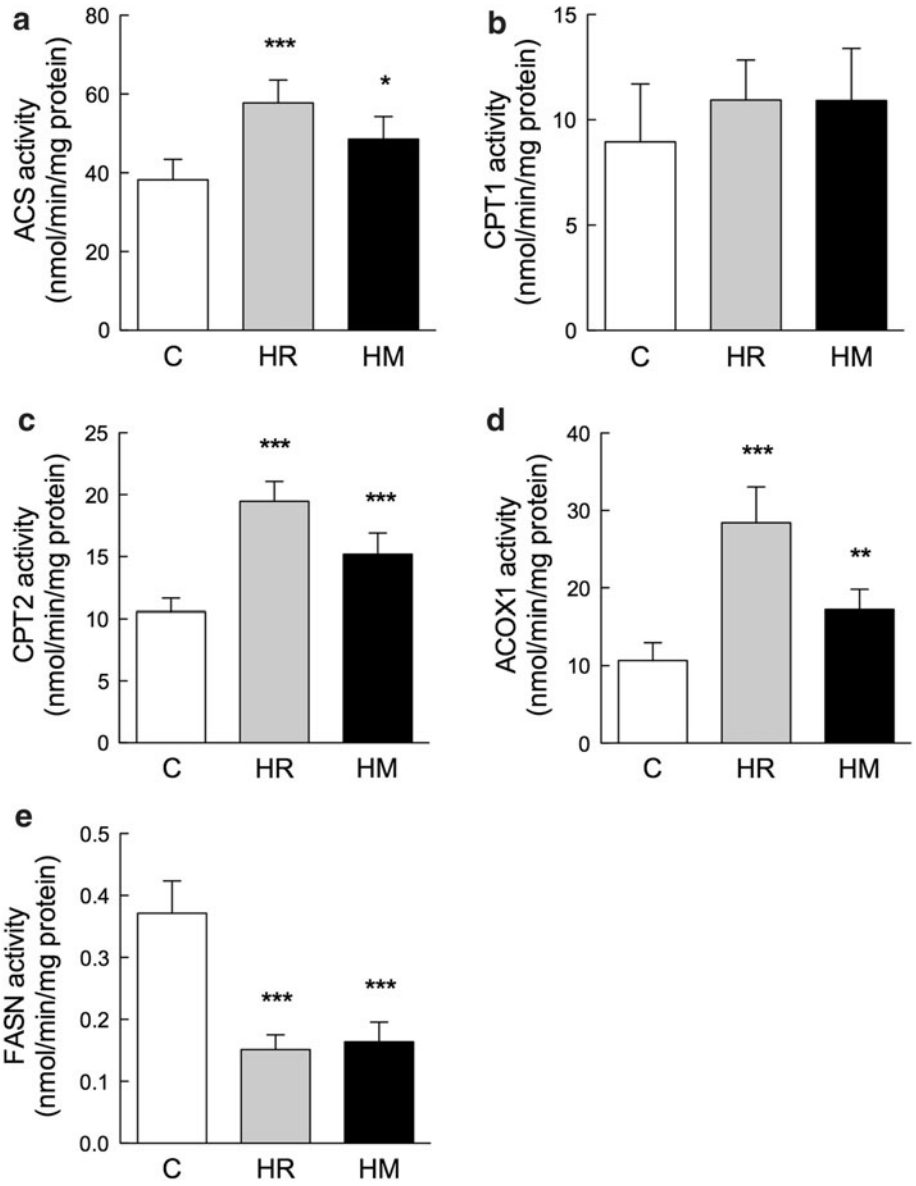

Fig. 3 The effect of 2 weeks herring roe and milt diets on the hepatic enzyme activities of ACS **a**, CPT1 **b**, CPT2 **c**, ACOX1 **d**, and FASN **e** in hTNFα transgenic mice. Data are given as means ± SD ($n = 5$), and values significantly different from control are indicted ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). *C* control, *HM* herring milt, *HR* herring roe, *ACS* acyl-CoA syntetase, *ACOX1* acyl-CoA oxidase 1, *CPT* carnitine palmitoyl transferase, *FASN* fatty acid synthase

accumulation in herring fed mice, as the hepatic gene expression of the TAG lipase AADAC was unchanged (Table 7) [33]. Herring roe and milt decreased the hepatic mRNA level of the LDL receptor. Nonetheless, cholesterol was increased in both plasma and liver, most likely due to the higher cholesterol level in the herring diets compared to control (Table 4). Interestingly, in $Ldlr^{-/-}$ mice, herring diets for 16 weeks gave a reduction in cholesterol levels both in plasma and in liver, possibly through reduced cholesterol synthesis and increased bile acid synthesis [34]. In agreement with this, we saw reduced expression of *Hmgcs1*, involved in cholesterol synthesis, and for herring milt only a significant increase in *Cyp7a1* expression, the rate-limiting enzyme in bile synthesis from cholesterol. Thus, there was an increase in hepatic cholesterol despite reduced expression of genes involved in cholesterol uptake and production in the herring groups. Among the three groups, the herring milt group had the lowest level of *Hmgcs1* expression (71% reduction vs. control) and the highest level of *Cyp7a1* expression (38% increase vs. control); nevertheless, this group paradoxically displayed the highest content in hepatic cholesterol (Fig. 1d). A possible cause may be the high cholesterol level in milt (which is 2 times more elevated compared to roe) (Table 4). Thus, the observed downregulation of genes involved in cholesterol production and upregulation of genes involved in bile formation could be compensatory. In contrast, the increase in plasma cholesterol was higher in the roe group than the milt, perhaps reflecting the differences in cholesterol synthesis and bile formation. We have previously shown that in this mouse model of persistent low-grade TNFα expression, the hypolipidemic PPAR

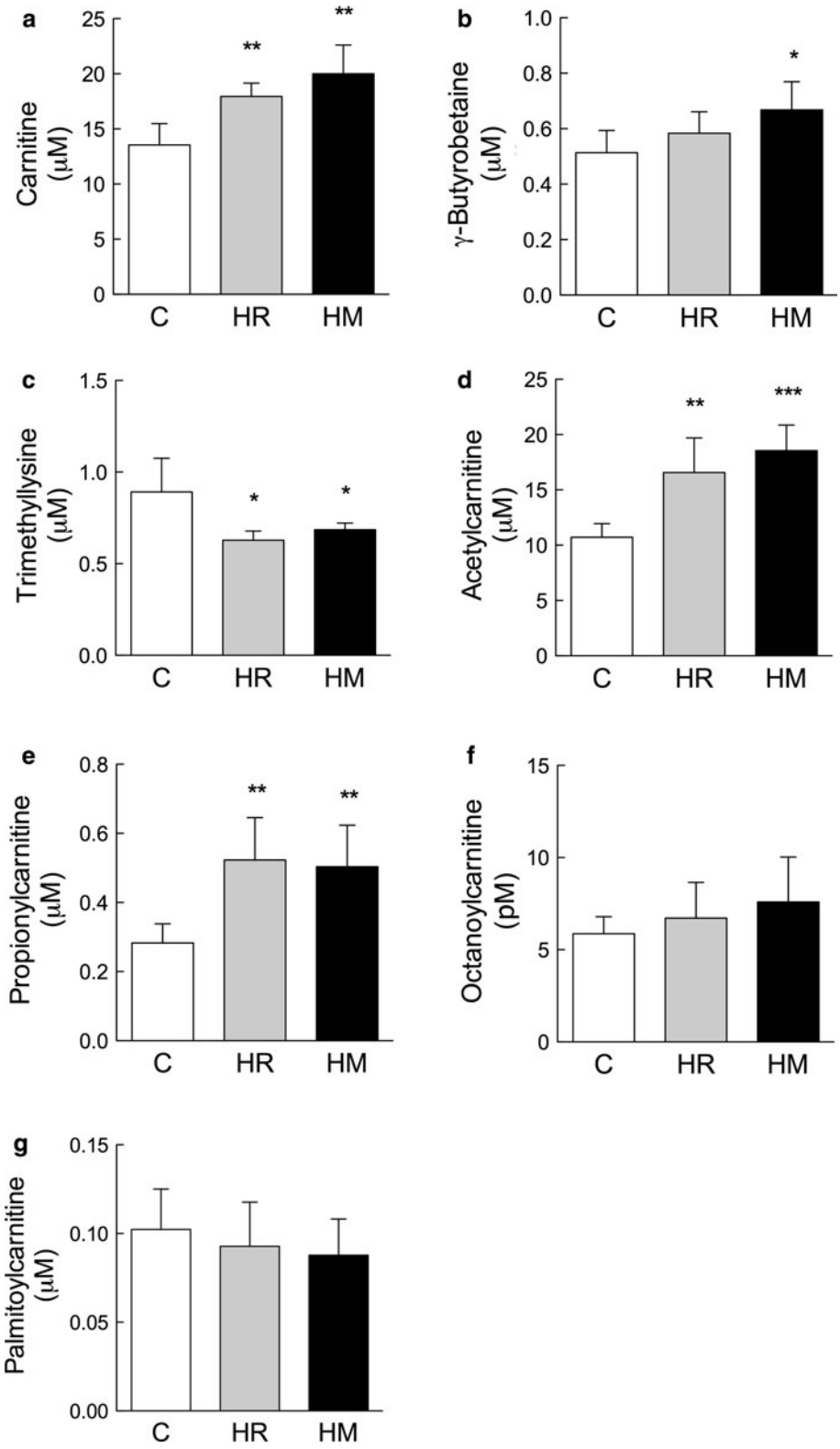

Fig. 4 Plasma levels of free carnitine **a**, γ-butyrobetaine **b**, trimethyllysine **c**, acetylcarnitine **d**, propionylcarnitine **e**, octanoylcarnitine **f**, and palmitoylcarnitine **g** after herring roe and milt treatment. Data are given as means ± SD ($n = 5$), and values significantly different from control are indicted ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). *C* control, *HM* herring milt, *HR* herring roe

agonist tetradecylthioacetic acid was unable to reduce hepatic lipid levels after 2 weeks of treatment [35], while 6 weeks of treatment reduced both plasma and hepatic TAG (data not shown). This suggests a severe disruption of hepatic lipid metabolism in the hTNFα mice and a delay in the response to PPAR activation. Thus, due to their high cholesterol content, herring diets could aggravate the disruption of cholesterol metabolism in hTNFα mice.

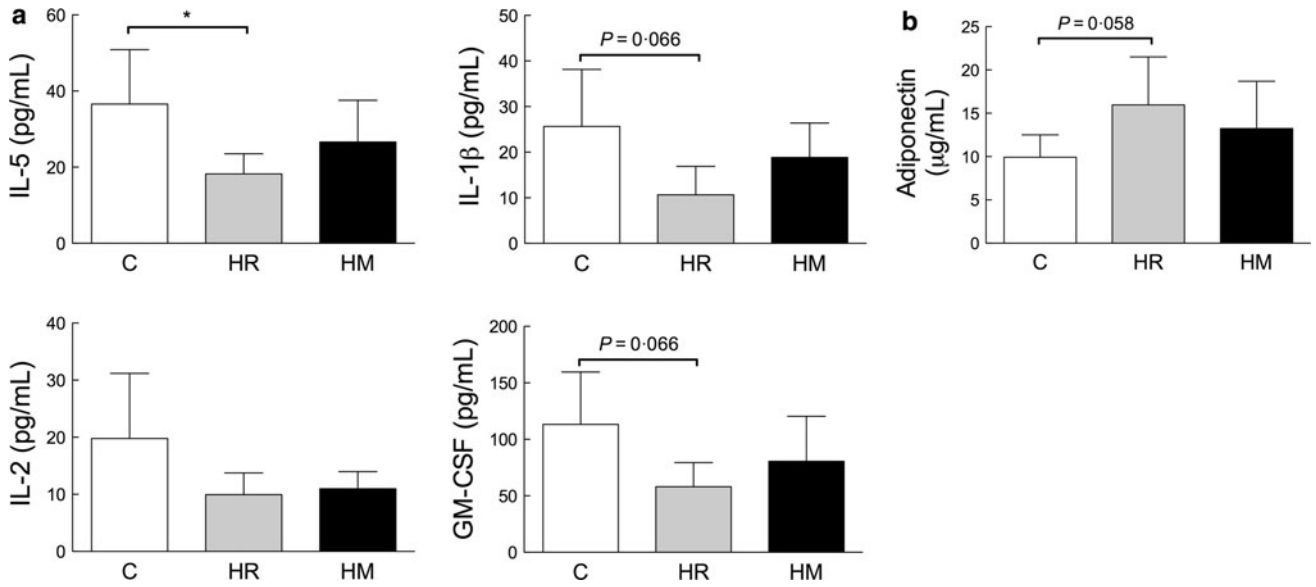

Fig. 5 Plasma cytokine levels of IL-1β, IL-2, IL-5, and CSF2 **a**, and the plasma adiponectin level **b** in response to 2 weeks herring roe or milt diets. Data are given as means ± SD ($n = 5$), and values significantly different from control are indicted (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). *C* control, *HM* herring milt, *HR* herring roe

However, it is not excluded that a longer treatment period with roe and/or milt could improve plasma and hepatic TAG levels.

Both diets contained high levels of EPA and DHA, mainly in the form of PC (Tables 3, 4), and some of the effects observed could be due to PPARα activation by lipid ligands, as indicated by the activation of PPAR-regulated genes. Moreover, the herring roe and milt are a rich source of fish protein, and recent evidence indicates that fish protein lowers plasma lipid levels and has effects on cholesterol metabolism involving regulation of bile acids [10, 12, 36]. Some discrepancies exist; in Wistar rats, a fish protein was demonstrated to reduce hepatic cholesterol concomitantly with increased bile acid excretion and *Cyp7a1* expression [36]. However, Sprague–Dawley rats fed 20% fish protein diets from Alaska Pollack showed reduced plasma TAG and HDL cholesterol, but increased hepatic cholesterol without altered CYP7A1 mRNA expression [11]. Importantly, protein from different types of fish has been shown to differentially affect energy metabolism and insulin sensitivity in rats [37].

By measuring plasma free carnitine and its acyl-esters, we looked at overall changes in the β-oxidation process. The formation of acylcarnitines is important to modulate the acyl-CoA/CoASH ratio in the cell. Surplus acyl-CoA will be transported out of the mitochondria or peroxisomes in the form of acylcarnitines. The plasma acylcarnitine level can thus be considered as a reflection of the intramitochondrial level of acyl-CoA. Even chain species of acylcarnitines that range from C6 to C22 reflect incomplete fatty acid oxidation. The odd chain species, such as propionylcarnitine (C3) and isovaleryl/valerylcarnitine (C5), are primarily derived from branched chain amino acid catabolism. Acetylcarnitine (C2) will reflect the level of acetyl-CoA, which is the end product of fatty acid oxidation, but can also result from amino acid or glucose metabolism. We found that plasma free carnitine levels were increased due to herring roe and milt feeding, as well as the short-chain carnitine-esters, namely acetylcarnitine and propionoylcarnitine, but not long-chain fatty acylcarnitines. A recent study in *Ppara*$^{-/-}$ mice showed that an impaired shift to β-oxidation during fasting results in a two- to threefold increase in the plasma long-chain acylcarnitines [38]. Carnitine deficiency and reduced tissue levels of short-chain acylcarnitine intermediates also accompanied fasting in *Ppara*$^{-/-}$ mice. Reversely, studies in male CD-1 mice indicated that treatment with PPAR activating agents will lead to an increase in plasma acetylcarnitine levels and a drop in plasma palmitoylcarnitine levels [39]. In accordance with these studies, we observed that fasted TNFα-mice on herring supplemented diets displayed increased plasma levels of short-chained acetylcarnitine and an insignificant reduction of long-chained palmitoylcarnitine compared to animals on a control high-fat diet. As we observed an increased hepatic lipid catabolism in the herring animals, it is likely that the accumulation of plasma acetylcarnitine reflects a surplus of acetyl-CoA-production in mitochondria.

The odd-chained propionylcarnitine is a product of branched amino acid (AA) degradation, and its increase suggested that the herring diets activated the branched chain AA-degradation pathway simultaneously with the

β-oxidation pathway. This is in accordance with PPARα-activation, since the numerous PPARα-target genes also include genes involved in the AA metabolism [40].

The increase in carnitine and the corresponding decrease in its precursor trimethyllysine suggest that carnitine catabolism is affected by the herring diets in TNFα-mice. Interestingly, PPARα activation may be necessary for an observed increase in hepatic carnitine storage in situations with increased levels of plasma acylcarnitines [38]. Thus, the increase in circulating free carnitine in the herring groups may reflect an increased lysine/trimethyllysine catabolism in the tissues.

The lipid contribution to the diet by herring roe and milt constituted 3.7 and 1.3% (w/w) of the diets, respectively (Tables 1, 2), and resulted in a 2- to 2.5-fold increase in *n*-3 PUFAs in liver and ovarian WAT (Table 6). Simultaneously, downregulation of the desaturases involved in PUFA synthesis, *Fads1* and *−2*, was observed in liver (Table 7), as well as a reduction in the Δ5 and Δ6 desaturase indexes in both liver and WAT (Table 6). We have previously shown that the proportions of fatty acids are affected in hTNFα mice compared to wt mice, resulting in increased saturated and PUFAs but decreased levels of monounsaturated fatty acids in liver [6]. Herring roe and milt diets counteracted this effect. MUFAs, in particular C18:1*n*-9, decreased in WAT but seemed to increase in the liver in the herring diet groups, perhaps due to an increase in MUFA-rich TAG and thus increase in the C18:1*n*-9/C18:0 ratio. A decreased Δ9 desaturase activity by the herring diets cannot be excluded, even if the hepatic *Scd1* gene expression level was unchanged, as indicated by the decreased C18:1*n*-9/C18:0 ratio in epididymal WAT. SCD1 mRNA was previously shown to be downregulated in TNFα mice compared to wild-type C57Bl/6 mice [6], resulting in a low SCD1 mRNA level that was however not prevented by the herring diets.

The effects of herring supplements on fatty acid composition and β-oxidation in TNFα-expressing mice were accompanied by a tendency to reduced plasma levels of the pro-inflammatory cytokines IL-5, IL-1β, IL-2, CSF2, and increased plasma adiponectin. In summary, herring roe and milt diets showed anti-inflammatory potential, increased mitochondrial and peroxisomal β-oxidation, reduced plasma TAG, enhanced plasma levels of short-chained acylcarnitines, and reversed the hTNFα-induced change in fatty acid composition in liver and ovarian WAT. This shows that herring roe and milt have the potential to ameliorate several of the negative effects of a high-fat diet and chronic inflammation in this mouse model. However, the herring diets aggravated the hepatic lipid accumulation in hTNFα mice. Further studies are entailed to investigate the health effect of herring roe and milt in humans.

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Conflict of interest The authors declare that they have no conflict of interest.

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