



# Effects of low doses of fish and milk proteins on glucose regulation and markers of insulin sensitivity in overweight adults: a randomised, double blind study

Ida Heir Hovland<sup>1</sup> · Ingrid Sande Leikanger<sup>1</sup> · Oddbjørg Stokkeland<sup>1</sup> · Kaia Hevrøy Waage<sup>1</sup> · Svein A. Mjøs<sup>2,3</sup> · Karl A. Brokstad<sup>4</sup> · Adrian McCann<sup>5</sup> · Per Magne Ueland<sup>6,7</sup> · Rasa Slizyte<sup>8</sup> · Ana Carvajal<sup>8</sup> · Gunnar Mellgren<sup>6,9</sup> · Tore Remman<sup>10</sup> · Ingmar Høggøy<sup>11</sup> · Oddrun A. Gudbrandsen<sup>1,12</sup> 

Received: 27 November 2018 / Accepted: 2 April 2019 / Published online: 10 April 2019  
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## Abstract

**Purpose** To examine whether supplementation with low doses of fish or milk proteins would affect glucose regulation and circulating lipid concentrations in overweight healthy adults.

**Methods** Ninety-three overweight adults were assigned to receive 2.5 g protein/day from herring (HER), salmon (SAL), cod (COD) or milk (CAS, a casein–whey mixture as positive control) as tablets for 8 weeks.

**Results** Seventy-seven participants were included in the analyses. HER and SAL did not affect glucose and insulin concentrations. COD significantly reduced within-group changes in 90 and 120 min postprandial glucose concentrations but changes were not different from HER and SAL groups. CAS supplementation significantly reduced the area under the curve for glucose concentrations (– 7%), especially when compared to SAL group, and reduced postprandial insulin c-peptide concentration (– 23%). Reductions in acetoacetate (– 24%) and  $\beta$ -hydroxybutyrate (– 29%) serum concentrations in HER group were more prominent compared to SAL and COD groups, with no differences between fish protein groups for  $\alpha$ -hydroxybutyrate. Serum concentrations of  $\alpha$ -hydroxybutyrate (– 23%), acetoacetate (– 39%) and  $\beta$ -hydroxybutyrate (– 40%) were significantly reduced within CAS group, and the decreases were significantly more pronounced when compared to SAL group. Serum lipid concentrations were not altered in any of the intervention groups.

**Conclusion** Findings indicate that 2.5 g/day of proteins from fish or milk may be sufficient to improve glucose regulation in overweight adults. The effects were most pronounced after supplementation with proteins from cod, herring and milk, whereas salmon protein did not affect any of the measurements related to glucose regulation.

**Clinical trial registration** This trial was registered at clinicaltrials.gov as NCT01641055.

**Keywords** Fish protein · Herring protein hydrolysate · Salmon protein hydrolysate · Cod protein · Milk protein · Glucose

## Introduction

Obesity is one of the greatest health problems worldwide, and overweight and obesity are attributed to about 2.8 million deaths each year [1, 2]. Obesity is strongly related to cardiovascular disease risk factors such as insulin resistance, type 2 diabetes mellitus (T2D), dyslipidemia and the

metabolic syndrome [1, 3]. Epidemiological studies suggest that intake of fish may protect against T2D [4–6], and intake of fish, mainly fatty fish, has also been found to beneficially influence body weight, blood lipids and glucose homeostasis, which could protect against T2D and have cardio-protective effect [5, 7–10]. Studies investigating the effects of cod intake in insulin-resistant adults have also reported improved insulin sensitivity and improved  $\beta$ -cell function [11].

Several possible markers of insulin sensitivity have been suggested, with the objective of identifying persons at risk of developing insulin resistance at an early stage. Amongst these,  $\alpha$ -hydroxybutyrate (a metabolite of threonine and methionine catabolism, and glutathione synthesis through the cysteine formation pathway) stands out as a

Ida Heir Hovland, Ingrid Sande Leikanger, Oddbjørg Stokkeland and Kaia Hevrøy Waage contributed equally to this work.

✉ Oddrun A. Gudbrandsen  
oddrun.gudbrandsen@k1.uib.no

Extended author information available on the last page of the article

promising early biomarker [12–14]. Also the ketone bodies  $\beta$ -hydroxybutyrate and acetoacetate have been associated with the risk of developing insulin resistance, possibly through increased production as a consequence of high circulating concentration of non-esterified fatty acid (NEFA) [14].

The positive health effects associated with fish intake have largely been attributed to long-chain n-3 PUFAs. However, fish is also a good source of proteins and contains all the essential amino acids, which are readily available as fish proteins are easy to digest [15]. It appears that dietary proteins are capable of exerting effects on the body that go beyond nutrient supply, effects that have been attributed to peptides and amino acids as bioactive substances. Rat studies suggest that proteins from various fish species exert different physiological effects such as improved insulin sensitivity or glucose tolerance [16–19], and reduced circulating concentrations of lipids [18, 20, 21]. Recently, we showed that in obese Zucker *fa/fa* rats, both salmon hydrolysate and cod protein feeding resulted in lower postprandial glucose concentration, herring hydrolysate feeding resulted in lower serum concentrations of HDL and LDL cholesterol, cod protein feeding resulted in lower serum fasting NEFA concentration, and rats fed proteins from herring or cod had higher ratio of n-3 to n-6 PUFA in serum when compared to casein–whey mixture [22, 23]. Results from these rat studies encouraged investigation of the same proteins in a clinical trial.

In the present study we investigated the effects of fish and milk proteins from the same production batches that were tested in obese Zucker *fa/fa* rats [22, 23]. Since we have previously shown that a daily dose of 6 g of cod protein improved glucose metabolism and lowered LDL cholesterol concentration in overweight and obese adults [24], we now wanted to test the potency of a lower daily dose of fish proteins. This study is considered to be a hypothesis generating pilot study, with the objective to investigate whether a low dose of 2.5 g of proteins from fish (herring, salmon and cod) or milk (casein–whey mixture) per day would be sufficient to affect glucose regulation and lipid metabolism in healthy overweight adults after 8 weeks intervention. Milk protein (a casein–whey mixture) was chosen as a positive control, since both casein and whey have been shown to stimulate insulin release and thereby suppress postprandial glucose [25–27].

## Methods

### Participants, study setting and ethics

The study population consisted of overweight adults of Caucasian origin. Participants were recruited from the region of Bergen through publicity in a local newspaper and the

intranet at Haukeland University Hospital in June–August 2012. Inclusion criteria were BMI > 27 kg/m<sup>2</sup>, fasting blood glucose < 7.0 mmol/L and 18–69 years of age. Exclusion criteria were pregnancy, breastfeeding, menopause, allergies to fish or milk, diagnosed diabetes mellitus, heart disease or gastrointestinal diseases, use of medications that could influence the metabolism of glucose and lipids, use of anti-inflammatory medications, intentional weight loss and/or large fluctuation in body weight (> 3 kg) over the previous 2 months, a consumption of more than 2–3 fish meals per 14 days, undertaking an extreme diet and/or excluding major food groups, and use of fish oil, n-3 PUFAs or multivitamin supplements. Participants with difficulties swallowing pills were advised not to participate in the study.

The study was designed as a double-blind, randomised, intervention study with a parallel group design, with four intervention arms; herring protein hydrolysate (HER), salmon protein hydrolysate (SAL), cod protein (COD) and casein–whey mixture as positive control (CAS). Participants consumed tablets in doses corresponding to a daily intake of 2.5 g protein for 8 weeks. Health professionals performing blood sampling and measuring body composition and height, and personnel conducting the laboratory analyses were blinded to group allocation.

Ninety-three persons were included in the study and were assigned to HER ( $N=23$ ), SAL ( $N=24$ ), COD ( $N=23$ ) or CAS ( $N=23$ ) groups. The participants were stratified into the different groups by the project manager on the basis of BMI, age and gender. All examinations were conducted at the Haukeland University Hospital, Bergen, Norway. Written information including a thorough description of the trial as well as the necessary instructions was emailed to the participants before trial start. To enhance compliance the participants were contacted by phone approximately 1 week prior to baseline and end point visits, during which they were informed of the schedule and procedures for the following visit. Also, a text message was sent 1–3 days before the 8-week visit, as a reminder of how to prepare for the upcoming visit. For any inquires during the trial period, members of the research group could be reached by email or telephone. Compliance was monitored through interviews; after one, four and 8 weeks intervention the participants were asked how many doses they had not taken since last contact, instead of asking how well they had complied, to lower the bar for reporting missing intake. As reward for completing the study, participants were offered a dietary consultation with a student dietician at the last visit and all the results from the analyses of blood samples.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Regional Ethics Committee of Western Norway (REC-number: 2011/572). Written informed consent was obtained from all

subjects. Participants were able to withdraw from the study at any time and no explanation was required. This trial was registered at clinicaltrials.gov as NCT01641055.

## Interventions

The study participants ingested 2.5 g of proteins from fish or milk per day from tablets for 8 weeks. The study was designed with four intervention groups, and the tablets contained proteins from herring, salmon, cod or casein–whey mixture (the latter group served as a positive control group). Tablets were produced by Faun Pharma AS (Vestby, Norway). The tablets were coded with numbers 1 through 4 by the manufacturer, hence the intervention was blinded for both participants and personnel involved in the trial. Amino acid composition in tablets were analysed in duplicates by Nofima BioLab (Fyllingsdalen, Norway) according to the method of Cohen and Michaud [28]. In brief, the samples were hydrolysed with 6N HCL at 110 °C for 24h followed by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. The amino acids derivatives were separated by reverse phase HPLC in a Waters 2695 Separation Unit, and read in a Waters 2475 Multi Florescence Detector with excitation at 250 nm and emission at 395 nm. Fatty acids in tablets were extracted by the method described by Bligh and Dyer [29] and methylated and analysed as described previously [22, 30–32]. The fish protein hydrolysates were difficult to compress into the tablets due to their fluffy texture, therefore, the protein content in HER and SAL tablets were 50% lower compared to that of COD and CAS tablets. In total, HER and SAL groups ingested 24 tablets per day whereas COD and CAS groups ingested 12 tablets per day to achieve a daily intake of 2.5 g of proteins from the intervention tablets. All tablets were coated and of the same size, texture and structure. Participants were instructed to swallow the tablets whole and ingest their daily dosage of tablets distributed in three equal dosages throughout the day. Participants were instructed to maintain their habitual lifestyle during the intervention, with an emphasis on consistent dietary intake and physical activity. This information was communicated to the participant in writing as well as orally.

Protein hydrolysates from fresh rest residual materials from Norwegian spring spawning herring (Atlantic herring, *Clupea harengus*, from Grøntvedt Pelagic AS, Norway) and salmon (Atlantic salmon, *Salmo salar*, from SalMar ASA, Norway) were prepared using a mixture of Papain and Bromelain (1:1 w/w, Enzybel Intl.s.a., Belgium) for 1 h, as previously described [23]. Herring residuals consisted of heads, guts and backbones after filleting, and salmon residuals was comprised of backbones after filleting. The herring and salmon protein hydrolysates contained peptides mainly < 4000 g/mol (constituting 96.7 and 92.7% of total peptides, respectively). The amounts

of peptides of size 200–500 g/mol (mainly di- and tripeptides) were similar in the two protein hydrolysates (herring, 14.3%; salmon, 14.0%) [23]. Cod protein (No 0271, Seagarden AS, Norway) was made from cooked, dried and micro milled muscle (Atlantic cod, *Gadus morhua*), and the casein–whey mixture (90% casein, 10% whey) used as control protein was purchased from KAPA JP (Armor Proteines, France). Cod protein and casein–whey mixture were not hydrolysed prior to use.

## Protocol for study visits

The total study period was 8 weeks, with study visits at baseline and after 8 weeks (endpoint) at the Clinical Research Unit at Haukeland University Hospital (Bergen, Norway) in August to November 2012. Examinations were conducted in the morning after an overnight fast. Participants were instructed not to eat or drink anything except water, or use substances containing nicotine after 22.00 the previous day, and to avoid strenuous physical exercise and alcohol intake for 24 h preceding the visits.

Body height was measured at baseline, using a wall-mounted stadiometer (Seca 222, Hamburg, Germany). Body weight and body composition were measured using a bioelectrical impedance analysis device (InBody 720, Biospace Co Ltd, Seoul, Korea) at baseline and endpoint in a fasting state. Producer's guidelines for use were followed, and participants were weighed barefoot before blood sampling, wearing light clothing and with an empty bladder.

Blood samples were collected at baseline and end point. Blood was drawn from an antecubital vein by inserting a cannula connected to a three-way tap for repeated measures. The system was flushed with sterile saline before and after each blood sample. Fasting blood were collected in Vacuette K2EDTA tubes (Greiner Bio-one, Austria) for collection of whole blood and isolation of plasma, and in Vacuette Z Serum Clot Activator Tubes (Greiner Bio-one, Austria) for isolation of serum.

To test the glucose tolerance participants were served a standardised breakfast meal containing fat and protein in addition to carbohydrates. The breakfast was served immediately after the collection of fasting blood samples, and consisted of 0.28 L orange juice, 75 g white bread, 10 g margarine, 20 g white cheese and 25 g strawberry jam, providing a total of 502 kcal (73 g carbohydrates, 14 g protein and 16 g fat). The meal had to be consumed within 15 min. The macronutrient and energy content in the breakfast were calculated using 'Mat på Data 5.1' [33]. Blood was collected for isolation of serum in the fasting state and at 30, 60, 90 and 120 min following the meal tolerance test at baseline and after 8 weeks.

## Estimation of energy and macronutrient intakes from dietary records

Participants completed dietary records of the 5 preceding days including at least 1 weekend-day before the baseline and the 5 preceding days before the 8-week visits. The intakes of energy, carbohydrates, proteins and fats were calculated from the participants' dietary record using the 'Mat på Data 5.1' software [33]. Food records were checked for completeness at both study visits.

## Estimation of physical activity

To control for changes in physical activity the participants registered the amount of physical activity undertaken in the 2 weeks preceding the baseline and 8-week visits, using a standardised form based on validated methods [34, 35]. Type, duration and the intensity of the activity were recorded. The standardised form included ten main categories of activities, and the intensity was described using the Borg scale [36]. The amount of physical activity was described using Metabolic Equivalents Task (MET); a physiological measurement that expresses the energy cost for an activity [34], for calculation of MET-time.

## Analyses in serum, plasma and whole blood

Analyses of glucose, total cholesterol, HDL cholesterol, LDL cholesterol and triacylglycerol in serum samples were performed by accredited methods using a Modular P instrument (Roche Diagnostics GmbH, Mannheim, Germany) at the Laboratory of Clinical Biochemistry at Haukeland University Hospital (Bergen, Norway). Analyses of insulin and insulin c-peptide in serum were performed by routine methods using the Immulite 2000 Immunoassay System (Siemens Healthcare GmbH, Erlangen, Germany) at the Hormone Laboratory at Haukeland University Hospital. Concentrations of leptin and adiponectin were measured in fasting EDTA plasma using xMAG<sup>®</sup>, a bead based technique (Luminex Corp., Austin, TX.), with the adipokine panels containing adiponectin (HADK1MAG-61K, EMD Millipore, EMD Millipore Corporation, Billerica, USA) and leptin (#HADK2MAG-61K) and were analysed on the Luminex 100 instrument (Luminex Corp., Austin, TX) with STarStation v.3 software (Applied Cytometry, Dinnington, Sheffield, UK). Serum NEFA was analysed on the Cobas c 111 system (Roche Diagnostics GmbH, Marburg, Germany) using the NEFA FS kit (Diagnostic Systems, Holzheim, Germany). HbA1c in whole blood was analysed on the Cobas c 111 system using the A1C-3 kit with A1CD2 haemolysing reagent (Roche Diagnostics GmbH, Marburg, Germany) for Cobas c111. The area under the curve (AUC) for five glucose measurements with 30 min intervals at each visit

was calculated using the trapezoid rule [37]. Fructosamine and albumin were measured in fasting serum using the FRA kit for Cobas c systems from Roche Diagnostics and the ALB2 kit for Cobas c111, both were measured on the Cobas c 111 system and results are presented as fructosamine/albumin ratio. Serum concentrations of  $\alpha$ -hydroxybutyrate,  $\beta$ -hydroxybutyrate and acetoacetate were measured by Bevitall AS (Bergen, Norway, <https://www.bevital.no>) using gas chromatography with tandem mass spectrometry, as previously described [38]. The method was upgraded to include  $\alpha$ -hydroxybutyrate,  $\beta$ -hydroxybutyrate and acetoacetate by adding ion-pairs for these analytes and authentic isotope-labelled internal standards to existing assays.

## Analysis of fatty acid composition in fasting serum

Serum samples were added to heneicosanoic acid as internal standard and were methylated without prior extraction of lipids, as described previously [30]. After methylation, lipids in the samples were extracted twice with iso-octane. The methyl esters were quantified by an Agilent 7890 gas chromatograph equipped with flame ionization detector and a BPX-70 capillary column as described in [31] with minor adjustments of the temperature programme. The compounds were identified by gas chromatography–mass spectrometry using the BPX-70 column and methodology as described in [32].

## Outcome measurements

The primary outcome of the present study was changes in serum fasting and postprandial glucose concentrations after a daily intake of 2.5 g protein from herring, salmon, cod or milk from supplements for 8 weeks. Secondary outcomes were changes in serum insulin, insulin c-peptide, NEFA, lipids, adiponectin, leptin, fatty acid composition and markers of insulin sensitivity.

## Sample size

The present study is considered to be a pilot study, since to our knowledge this is the first study on the effects of a low dose (2.5 g/day) of protein from fish (herring, salmon or cod) or milk (a casein–whey mixture) on glucose regulation and lipid metabolism in overweight adults. As there is no information available about the effect size for a low dose of 2.5 g protein per day, we consider the present study to be hypothesis-generating rather than hypothesis-testing, and the study will contribute to sample size calculations for future studies with similar designs.

## Statistical analyses

Statistical analyses were conducted using SPSS Statistics 25 (SPSS, Inc., IBM Corporation, Armonk, NY, USA). Subjects that did not complete the study were excluded from laboratory and statistical analyses. Most biological and biochemical data were not normally distributed according to the Shapiro–Wilk test, therefore, non-parametric tests were used to investigate changes within groups (Wilcoxon Signed Ranks Test) and Kruskal Wallis Test was used to compare values between the four groups at baseline. These data are presented as medians with interquartile ranges. Energy and macronutrient intakes and physical activity were normally distributed, and changes within each group were tested using the Paired-Samples *t* Test and variables were compared between groups using One-Way Analysis of Variance (ANOVA). These data are presented as means with standard deviation. CAS group was included as a positive control group since effects of milk proteins on glucose regulation have been reported by others [25–27], and changes within the fish protein groups (HER, SAL and COD) were compared to each other and to milk protein group (CAS) using Mann Whitney Test. Data were not corrected for multiple testing post hoc, in line with the recommendation by Streiner [39] for variables that are not independent of each other.

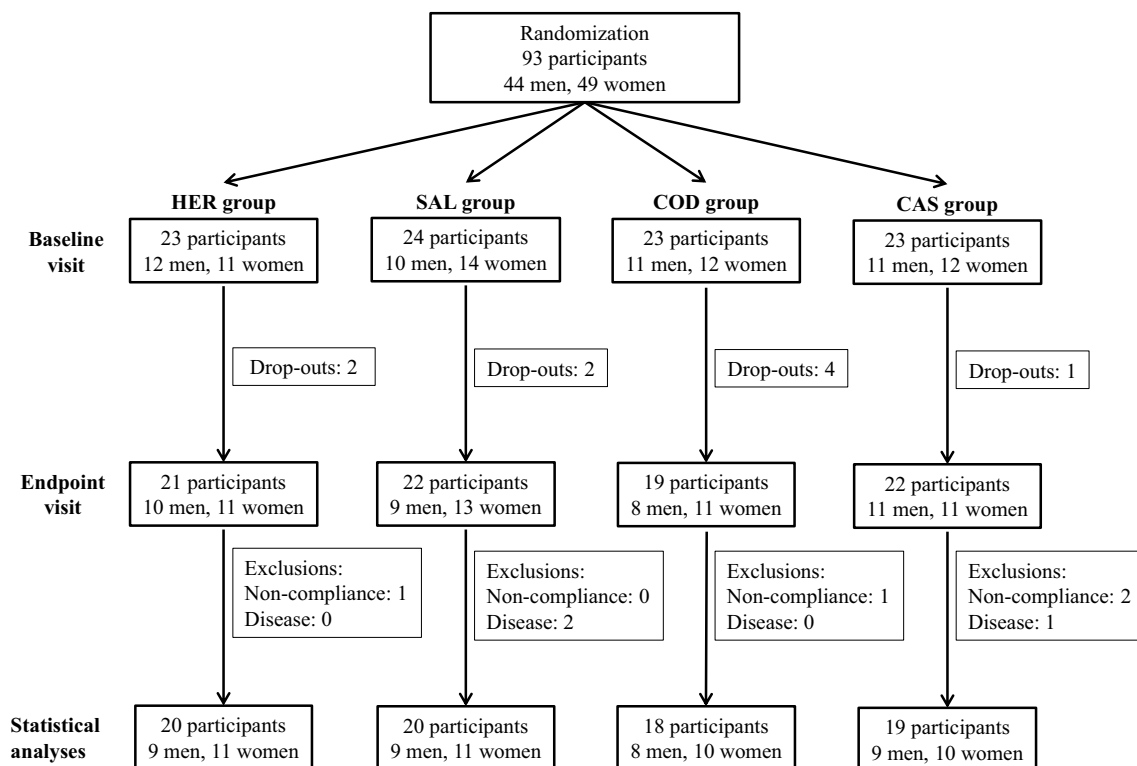
Gender distribution in the groups was compared using Pearson Chi-Square test. All comparisons were two-sided, and  $P < 0.05$  was considered statistically significant.

## Results

### Participant characteristics

Ninety-three participants were included in the study and completed the first study visit, and 84 participants (> 90%) completed the trial. Of the 84 participants, seven were excluded from statistical analyses; one participant (a man, in the HER group) was excluded from statistical analyses after it was revealed that he had hypertriglyceridemia, three participants (two women in the SAL group and one woman in the CAS group) were ill at the endpoint visit, and three participants were withdrawn from analysis because they did not comply with the protocol (one woman in the COD group and two men in the CAS group). In total, 77 subjects (35 men and 42 women) were included in the statistical analyses. Figure 1 shows the flow of participants through the study.

Participants were overweight or obese (median BMI 32.2 (range 29.9–35.7) kg/m<sup>2</sup>), with median age 41.9 (range 35.4–49.1) years at baseline. Baseline characteristics for



**Fig. 1** Study overview of participants. Participants not complying with the protocol were not included in the statistical analyses. Non-compliance was defined as not following the protocol in regard to

taking the tablets as instructed and a weekly fish intake above the accepted level, and newly diagnosed diseases and/or use of prescription medicine that are not compatible with the inclusion criteria

the groups are presented in Table 1. Groups were similar at baseline for gender distribution, age, BMI, body fat and muscle mass. Four participants reported that they were daily cigarette smokers; one in each experimental group, all the smokers were women and none of them were heavy smokers (defined by the World Health Organization as smoking 20 or more cigarettes per day [40]). After 8 weeks no changes were seen in anthropometric data in any of the groups (data not presented). All included participants had HbA1c < 6.5% (< 48 mmol/mol IFCC) at baseline.

### Energy and macronutrient intakes and physical activity

Estimated dietary intakes of energy, protein, fat and carbohydrates were similar between the groups at baseline, and intakes were not changed from baseline visit to 8-week visit within any of the groups (Table 2). We found no significant differences in the estimated volume of physical activity between the groups at baseline and no significant changes in volume of physical activity (calculated MET-time) from baseline to endpoint for any group ( $p$  ANOVA = 0.77, data not presented).

### Amino acids and n-3 PUFAs in intervention tablets

The compositions of the tablets including stabilization and filling agents (microcrystalline cellulose, tricalcium phosphate and magnesium stearate) are presented in Table 3.

The HER and SAL tablets contained hydrolysed proteins from residual materials from herring and salmon, whereas COD tablets contained unhydrolysed protein from cod muscle. CAS tablets contained unhydrolysed proteins from a casein–whey mixture (9:1 ratio). The daily amino acid intake from tablets was in general very similar between HER and SAL groups, with the exception of the much higher arginine in HER tablets (Table 4). The amino acid composition in COD tablets differed from that in HER and SAL tablets, especially with regard to higher content of the essential

amino acids isoleucine, leucine, lysine, methionine, phenylalanine and valine, the conditionally essential amino acid tyrosine, and the non-essential amino acids aspartic acid and glutamic acid, but lower content of glycine, hydroxyproline and proline in the COD tablets. The amino acid composition in the CAS tablets, containing milk protein, was different from the fish protein tablets with regard to most amino acids. Notably, CAS tablets had higher content of essential amino acids histidine, isoleucine, leucine, phenylalanine, threonine and valine, as well as higher content of glutamic acid, proline and tyrosine, and lower content of glycine, arginine and alanine. Long-chain n-3 PUFAs were found only in COD tablets, and daily dose was 12 mg EPA, 2 mg DPA and 31 mg DHA.

### Glucose homeostasis

Fasting serum concentrations of glucose, insulin and insulin c-peptide were not changed within any of the experimental groups from baseline to 8 weeks (Table 5).

Postprandial serum concentrations (relative to fasting concentration) of glucose, insulin and insulin c-peptide were not significantly changed in HER and SAL groups after 8 weeks intervention (Table 5, Fig. 2). In the COD group, the differences between fasting and both 90 and 120 min postprandial serum glucose concentrations were significantly reduced after 8 weeks ( $p$  values 0.027 and 0.0017, respectively), with no significant change in AUC-glucose and with no changes in fasting to 120 min postprandial insulin and insulin c-peptide concentrations. When fish protein groups were compared, no differences were seen in fasting concentrations or in changes from fasting to postprandial concentrations of glucose, insulin and insulin c-peptide.

Postprandial glucose profile was markedly affected in CAS group, with significantly lower glucose (relative to fasting concentration) measured at 30, 60, 90 and 120 min after breakfast ( $p$  values 0.026, 0.033, 0.00030 and 0.014, respectively), and concomitant with this, AUC-glucose was significantly reduced after 8 weeks intervention. When changes in

**Table 1** Participant characteristics at baseline (medians and 25th, 75th percentiles)

	HER group ( $N=20$ )		SAL group ( $N=20$ )		COD group ( $N=18$ )		CAS group ( $N=19$ )		$P^*$
Men/women	9/11		9/11		8/10		9/10		0.98
Age (years)	39.3	34.7, 44.2	41.3	35.7, 50.0	43.0	36.2, 55.5	42.2	34.2, 48.7	0.43
Body weight (kg)	97.6	84.5, 110.1	101.6	90.1, 113.9	98.5	83.0, 104.5	100.5	91.4, 110.2	0.22
BMI ( $\text{kg}/\text{m}^2$ )	33.5	30.0, 35.3	33.8	31.0, 37.1	30.8	28.8, 32.6	32.3	30.1, 36.6	0.14
Body fat (%)	39.8	29.7, 41.6	40.3	28.6, 47.6	34.1	27.3, 43.7	40.2	33.7, 45.7	0.62
Muscles (%)	33.6	29.2, 40.2	33.5	28.9, 40.8	36.7	31.3, 41.9	33.8	30.3, 37.8	0.66

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

\*Groups were compared at baseline using Pearson Chi-square (categorical data) or Kruskal–Wallis test (continuous data)

**Table 2** Energy and macronutrient intakes (means and standard deviations)

Intervention groups	Baseline		8 weeks		<i>p</i> <sup>†</sup>	<i>p</i> <sup>‡</sup>
	Mean	SD	Mean	SD		
Energy (kcal/day)						
HER	2061	524	1956	490	0.42	0.38
SAL	2101	465	1934	643	0.17	
COD	1930	387	2004	499	0.54	
CAS	1856	525	1894	478	0.25	
Protein (g/day)						
HER	99	27	86	19	0.072	0.63
SAL	88	20	82	25	0.31	
COD	90	21	89	20	0.78	
CAS	85	25	82	15	0.44	
Total fat (g/day)						
HER	87	26	77	22	0.12	0.15
SAL	90	20	90	23	0.39	
COD	82	22	88	24	0.43	
CAS	67	21	74	19	0.077	
Carbohydrates (g/day)						
HER	202	65	203	68	0.95	0.36
SAL	206	58	192	77	0.25	
COD	180	55	196	59	0.19	
CAS	192	50	190	51	0.84	

No differences were observed between the groups at baseline (one-way analysis of variance (ANOVA)). Results are presented for twenty participants in the HER group, twenty participants in the SAL group, eighteen participants in the COD group and nineteen participants in the CAS group

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

<sup>†</sup>Within-group changes are tested using the paired-samples *t* test

<sup>‡</sup>Changes within groups are compared using one-way ANOVA

**Table 3** Description of intervention tablets

	HER	SAL	COD	CAS
Protein meal*, mg/tablet	237	237	237	232
Microcrystalline cellulose, mg/tablet	119	119	119	119
Tricalcium phosphate, mg/tablet	47	47	47	47
Magnesium stearate, mg/tablet	99	99	99	99
Number of tablets eaten, per day	24	24	12	12
Protein from tablets, mg/day	2503	2503	2503	2506

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

\*Crude protein contents in protein meals were as follows; herring protein hydrolysate, 44%; salmon protein hydrolysate, 44%; cod protein, 88%; casein–whey mixture, 90%

postprandial differences from fasting to 30, 60 and 90 min glucose and the change in AUC-glucose from baseline to 8 weeks were compared between fish protein groups and CAS group, the change within CAS group was significantly lower only when compared to SAL group (*p* values 0.010, 0.027, 0.028 and 0.021, respectively). The change from fasting to 120 min insulin c-peptide was significantly reduced

after 8 weeks in the CAS group, but this was not statistically significant when compared to the fish protein groups. Also, no change was seen in 120 min postprandial relative to fasting insulin concentration within or between the experimental groups.

### Markers of glucose regulation and insulin sensitivity

Serum concentrations of  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate were reduced within both HER and CAS groups, and in addition serum  $\beta$ -hydroxybutyrate concentration and fructosamine/albumin ratio were reduced in COD group after 8 weeks intervention (Table 6). SAL supplementation did not affect any of these markers of glucose regulation.

The decreases in  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate were similar between HER and CAS groups, whereas the decrease in these parameters were significantly more pronounced in CAS group when compared to SAL group. For COD group, the decrease in  $\alpha$ -hydroxybutyrate was less pronounced and the decrease in fructosamine was more pronounced than in CAS group,

**Table 4** Amino acid content of intervention tablets, presented as daily dosage

Amino acids (mg/day)	HER	SAL	COD	CAS
Alanine	164.2	178.3	151.9	88.3
Arginine	280.6	156.8	167.3	98.0
Aspartic acid + asparagine	180.6	210.7	267.5	197.9
Glutamic acid + glutamine	284.6	317.5	397.6	576.5
Glycine	217.0	281.2	133.5	50.6
Histidine	54.5	67.0	62.5	74.7
Hydroxyproline	30.7	57.4	9.08	<LOD
Isoleucine	69.8	81.8	127.2	147.1
Leucine	136.3	144.3	212.2	263.8
Lysine	184.6	175.5	261.2	236.3
Methionine	51.7	64.2	86.6	77.2
Phenylalanine	64.8	72.1	104.5	135.8
Proline	147.1	168.1	101.1	262.4
Serine	125.5	106.2	117.6	148.8
Threonine	115.9	107.4	118.1	143.7
Tyrosine	42.6	42.0	72.4	107.1
Valine	104.5	105.6	133.8	176.1

Means of two measurements, deviations were less than 5% between parallels

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture, LOD level of detection

while the reductions in acetoacetate and  $\beta$ -hydroxybutyrate were similar to CAS group. Fish protein groups were similar in regard to changes in  $\alpha$ -hydroxybutyrate and fructosamine, however, the reductions in acetoacetate and  $\beta$ -hydroxybutyrate were more prominent in HER group compared to SAL and COD groups, with no differences between the two latter groups.

### Leptin, adiponectin and NEFA

After 8 weeks, the fasting adiponectin concentration was increased in the COD group, whereas the fasting concentration of leptin was significantly increased in the CAS group, with no changes from baseline to 8 weeks in the other groups (Table 7). When within-group changes were compared between fish protein groups and CAS group for leptin and adiponectin, a significant difference was evident only in the change in leptin concentrations between COD and CAS group. For comparisons between fish protein groups, no differences were seen between groups for changes in leptin and adiponectin.

Fasting NEFA concentration was not affected by fish protein intake, but was markedly reduced in the CAS group (Table 7). The within-group changes in fasting NEFA from baseline to endpoint were significantly different for all fish protein groups when compared to CAS group. Postprandial NEFA concentrations measured after 8 weeks intervention

were significantly reduced in CAS group after 30 min ( $p = 5.3 \times 10^{-4}$ ), 60 min ( $p = 0.026$ ) and 90 min ( $p = 0.041$ ), and this was significantly lower when compared to SAL group but was not significantly different from COD and SAL groups. Postprandial NEFA concentrations were not affected in HER, SAL and COD groups, with no differences between the fish protein groups for changes in postprandial NEFA concentrations (data not presented).

### Fatty acids and lipids in serum

The complete fatty acid profile was analysed in fasting serum samples collected at baseline and endpoint, and total amounts of SFA, MUFA, n-3 PUFA and n-6 PUFA, as well as n-3/n-6 PUFA ratio, were calculated. The results show that the total amount of SFA was increased in HER and SAL groups and the total MUFA was reduced in CAS group, and otherwise no within-group changes were observed for total SFA and MUFA and no differences were seen between the groups (Table 8). In HER group, total n-3 PUFA and n-3/n-6 PUFA ratio were significantly reduced with no change in total n-6 PUFA in serum from baseline to 8 weeks, due to lower amounts of EPA (20:5n-3) and DPA (22:5n-3), with  $p$  values 0.019 and 0.035, respectively (data not presented). In SAL, COD and CAS groups, no changes were seen in total amounts of n-3 and n-6 PUFAs or in n-3/n-6 PUFA ratio. When fish protein groups were compared to CAS group, no differences were seen between groups for changes in PUFAs. Also, no differences were seen for changes in PUFAs between fish protein groups.

Fasting serum concentrations of triacylglycerols, total cholesterol, HDL cholesterol and LDL cholesterol were not changed from baseline to endpoint within any of the groups, and no between-group differences were observed when fish protein groups were compared to CAS group or between fish protein groups (data not presented).

### Discussion

The main objective of the present study was to assess whether a low daily intake (2.5 g/day) of herring protein hydrolysate (HER), salmon protein hydrolysate (SAL), cod protein (COD) or a casein–whey mixture (CAS) from supplements for 8 weeks would affect glucose regulation and lipid metabolism in healthy overweight adults. Here, we show for the first time that a low daily dose of 2.5 g protein from herring, cod or milk may be sufficient to improve postprandial glucose metabolism as indicated by changes in postprandial concentrations of glucose, insulin and/or NEFA, or fasting concentrations of  $\alpha$ -hydroxybutyrate, acetoacetate,  $\beta$ -hydroxybutyrate and/or fructosamine in overweight healthy adults, while protein hydrolysate from salmon did



**Table 5** Serum concentrations of glucose, insulin and insulin c-peptide (medians and 25th, 75th percentiles)

Intervention groups	Baseline		8 weeks		$P^{\dagger}$	$P^{\ddagger}$	$P^{\S}$
	Median	25th, 75th percentile	Median	25th, 75th percentile			
Fasting glucose (mmol/l)							
HER	5.2	5.0, 5.6	5.3	5.0, 5.6	0.89	0.86	0.33 <sup>A</sup>
SAL	5.4	4.9, 5.5	5.0	4.6, 5.7	0.44	0.40	0.28 <sup>B</sup>
COD	5.1	4.8, 5.5	5.2	5.0, 5.4	0.38	0.43	0.21 <sup>C</sup>
CAS	5.3	5.1, 5.7	5.3	5.0, 5.8	0.56		
$\Delta$ fasting to 120 min glucose (mmol/l)							
HER	0.3	– 0.3, 0.8	0.2	– 0.6, 0.4	0.59	0.10	0.87 <sup>A</sup>
SAL	– 0.1	– 0.6, 0.4	– 0.3	– 0.7, 0.0	0.79	0.092	0.17 <sup>B</sup>
COD	0.0	– 0.6, 0.6	– 0.4	– 0.9, 0.4	0.0017	0.64	0.095 <sup>C</sup>
CAS	0.0	– 0.5, 0.8	– 0.5	– 0.8, 0.1	0.014		
AUC-glucose (mmol/l/min)							
HER	773	699, 863	751	685, 840	0.31	0.22	0.32 <sup>A</sup>
SAL	716	645, 837	720	621, 847	0.67	0.021	0.70 <sup>B</sup>
COD	758	680, 823	731	649, 818	0.15	0.46	0.17 <sup>C</sup>
CAS	767	728, 831	708	645, 783	0.011		
Fasting insulin (pmol/l)							
HER	71.7	53.6, 96.6	77.1	54.0, 99.0	0.97	0.90	0.96 <sup>A</sup>
SAL	72.0	50.7, 89.3	57.6	38.4, 89.6	0.92	0.92	0.74 <sup>B</sup>
COD	69.6	29.3, 84.8	65.1	55.5, 84.0	0.59	0.68	0.17 <sup>C</sup>
CAS	76.2	56.4, 138.0	67.2	51.0, 140.4	1.00		
$\Delta$ fasting to 120 min insulin (pmol/l)							
HER	222.9	90.8, 336.2	198.3	93.3, 371.1	0.34	0.66	0.82 <sup>A</sup>
SAL	139.5	62.0, 234.3	103.2	60.8, 175.7	0.77	0.46	0.76 <sup>B</sup>
COD	132.9	95.1, 212.6	136.5	57.2, 218.6	0.65	0.25	0.69 <sup>C</sup>
CAS	231.0	91.8, 295.1	169.8	76.8, 261.0	0.15		
Fasting insulin c-peptide (nmol/l)							
HER	0.88	0.65, 1.11	0.86	0.63, 1.08	0.53	0.48	0.88 <sup>A</sup>
SAL	0.84	0.69, 0.94	0.78	0.59, 0.97	0.76	0.39	0.40 <sup>B</sup>
COD	0.80	0.60, 0.94	0.78	0.67, 1.02	0.55	0.81	0.91 <sup>C</sup>
CAS	0.81	0.66, 1.15	0.81	0.74, 1.24	0.63		
$\Delta$ fasting to 120 min insulin c-peptide (nmol/l)							
HER	1.7	1.0, 2.3	1.3	0.9, 2.3	0.067	0.42	0.40 <sup>A</sup>
SAL	1.2	0.7, 1.9	0.9	0.7, 1.3	0.53	0.10	0.46 <sup>B</sup>
COD	1.3	0.9, 1.8	1.4	0.7, 1.7	0.16	0.10	0.39 <sup>C</sup>
CAS	1.6	1.1, 2.3	1.2	0.8, 2.0	0.0035		

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

No differences were observed between the groups at baseline (Kruskal–Wallis test). Results are presented for twenty participants in the HER group, twenty participants in the SAL group, eighteen participants in the COD group and nineteen participants in the CAS group

<sup>†</sup>Within-group changes are tested using Wilcoxon's signed-rank test

<sup>‡</sup>Changes within fish protein groups are compared with the CAS group using the Mann–Whitney test

<sup>§</sup>Changes within fish protein groups are compared using the Mann–Whitney test

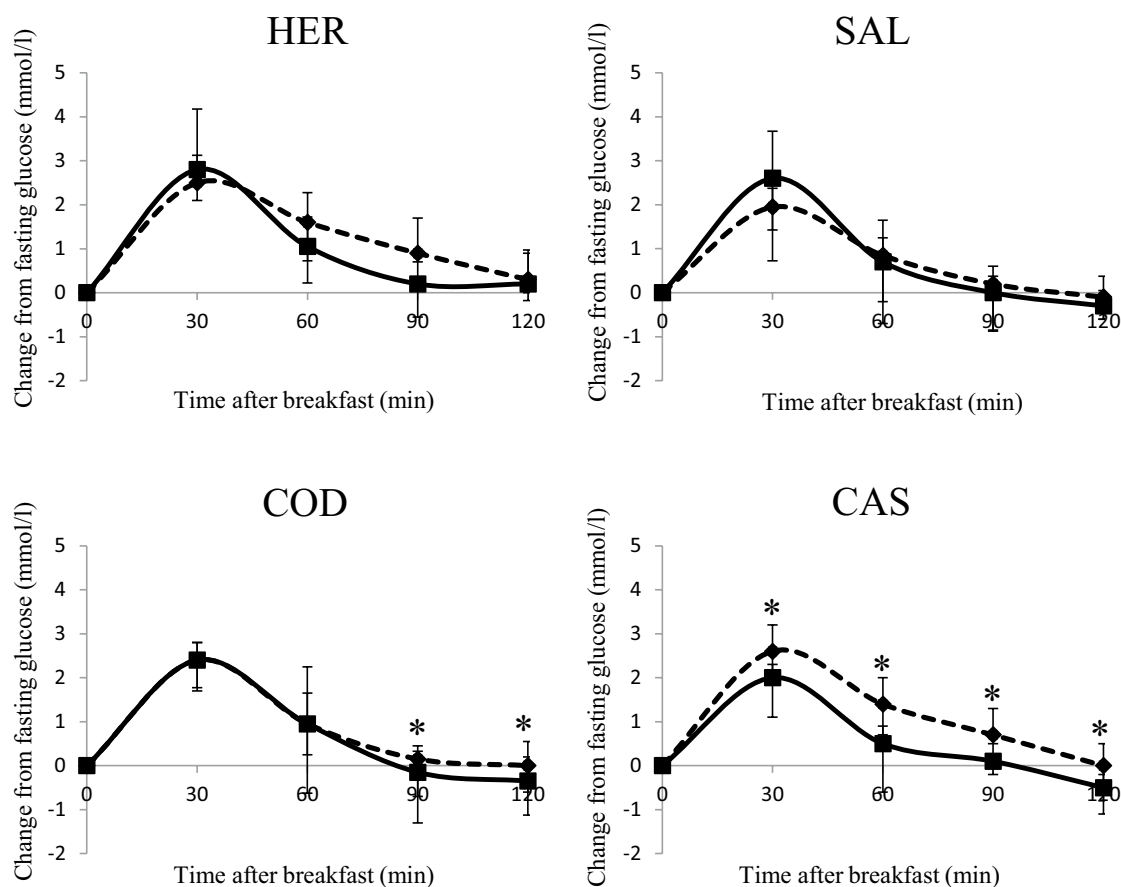
<sup>A</sup>HER group is compared to SAL group

<sup>B</sup>HER group is compared to COD group

<sup>C</sup>SAL group is compared to COD group

not affect any of the measured markers of glucose regulation. Neither fish proteins nor milk protein affected serum lipid concentrations.

Daily supplementation with 2.5 g of COD protein was sufficient to reduce postprandial concentrations of glucose, reduce fasting concentrations of fructosamine and



**Fig. 2** Glucose response after intake of a standardised breakfast in herring hydrolysate group (HER), salmon hydrolysate group (SAL), cod protein group (COD) and casein–whey group (CAS), shown relative to fasting glucose concentrations. Glucose response was measured at baseline (stippled line) and after 8 weeks (solid line). Results

are presented for twenty participants in the HER group, twenty participants in the SAL group, eighteen participants in COD group, and nineteen participants in the CAS group, and are presented as medians with 25th, 75th percentiles. \*Significant change from baseline to end-point within experimental group (Wilcoxon's signed-rank test)

$\beta$ -hydroxybutyrate and increase serum adiponectin concentration, with no change in insulin, insulin c-peptide, leptin and NEFA in serum. Although significant changes were found only within the COD group and did not show improvement when compared to the other fish protein groups and CAS group, the findings are suggestive of improved regulation of postprandial glucose concentrations, which is in line with our previous findings in overweight adults [24] and obese rats [22] after cod protein intake. Since the postprandial serum concentrations of insulin and insulin c-peptide were not affected by COD intake, improved insulin sensitivity may underlie the observation in this group.

HER supplementation reduced serum concentrations of  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate, which may be indicative of improved insulin sensitivity despite no overt changes in fasting and postprandial concentrations of glucose, insulin and insulin c-peptide. The reduction in  $\alpha$ -hydroxybutyrate was similar in HER and SAL groups, however, the reductions in acetoacetate and

$\beta$ -hydroxybutyrate in HER group were significantly greater than in COD and SAL groups. SAL supplementation did not affect fasting or postprandial concentrations of glucose, insulin and insulin c-peptide, or of any of the measured markers of insulin sensitivity. This was unexpected since in the Zucker *fa/fa* rat study, the salmon hydrolysate was more potent than the herring hydrolysate in terms of lowering postprandial glucose concentrations [23].

Milk proteins have been shown to be potent, acute stimulators of insulin secretion, and therefore, reduce postprandial blood glucose concentration, and inhibit both hormone-sensitive lipase and release of NEFA from adipose tissue [25–27]. CAS intake for 8 weeks profoundly lowered postprandial, but not fasting, concentrations of glucose. Although milk protein is recognised for being insulinotropic in acute experiments, we observed the opposite after 8 weeks intervention, as the lower postprandial concentration of insulin c-peptide suggests that insulin secretion from  $\beta$ -cells was lower and that insulin sensitivity was improved

**Table 6** Biomarkers of insulin sensitivity and fructosamine/albumin ratio in serum (medians and 25th, 75th percentiles)

Intervention groups	Baseline		8 weeks		$P^{\dagger}$	$P^{\ddagger}$	$P^{\S}$
	Median	25th, 75th percentile	Median	25th, 75th percentile			
$\alpha$ -hydroxybutyrate (umol/l)							
HER	47.2	37.3, 56.9	40.3	34.3, 52.8	0.048	0.18	0.12 <sup>A</sup>
SAL	38.9	31.1, 48.8	40.2	31.9, 49.1	0.97	$1.8 \times 10^{-3}$	0.56 <sup>B</sup>
COD	47.9	35.5, 57.7	41.8	34.8, 57.0	0.20	0.031	0.41 <sup>C</sup>
CAS	50.0	39.6, 54.4	34.6	30.7, 44.2	$3.4 \times 10^{-4}$		
Acetoacetate (umol/l)							
HER	48.2	34.1, 72.6	33.0	24.0, 47.8	0.030	0.59	0.0068 <sup>A</sup>
SAL	32.4	24.0, 57.1	31.5	27.0, 85.9	0.12	$6.4 \times 10^{-3}$	0.62 <sup>B</sup>
COD	49.5	28.7, 71.7	41.0	28.2, 57.4	0.10	0.13	0.028 <sup>C</sup>
CAS	43.2	37.2, 63.0	33.6	24.0, 47.3	$8.9 \times 10^{-3}$		
$\beta$ -hydroxybutyrate (umol/l)							
HER	58.6	41.3, 103.7	44.5	34.7, 61.5	0.025	0.38	0.016 <sup>A</sup>
SAL	45.6	34.6, 93.4	48.8	32.2, 139.7	0.31	$1.2 \times 10^{-3}$	0.58 <sup>B</sup>
COD	72.6	39.5, 94.7	50.7	35.7, 70.9	0.048	0.31	0.047 <sup>C</sup>
CAS	53.0	47.0, 104.7	36.1	29.5, 59.7	$7.2 \times 10^{-4}$		
Fructosamine (umol/g/albumin)							
HER	4.9	4.7, 5.2	5.0	4.6, 5.2	0.17	0.24	0.87 <sup>A</sup>
SAL	5.1	4.7, 5.4	5.0	4.7, 5.3	0.12	0.18	0.31 <sup>B</sup>
COD	5.2	4.9, 5.5	5.0	4.8, 5.3	0.039	0.040	0.58 <sup>C</sup>
CAS	5.1	4.8, 5.4	5.1	4.8, 5.3	0.72		

No differences were observed between the groups at baseline (Kruskal–Wallis test). Results are presented for twenty participants in the HER group, twenty participants in the SAL group, eighteen participants in the COD group and nineteen participants in the CAS group

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

<sup>†</sup>Within-group changes are tested using Wilcoxon's signed-rank test

<sup>‡</sup>Changes within fish protein groups are compared with the CAS group using the Mann–Whitney test

<sup>§</sup>Changes within fish protein groups are compared using the Mann–Whitney test

<sup>A</sup>HER group is compared to SAL group

<sup>B</sup>HER group is compared to COD group

<sup>C</sup>SAL group is compared to COD group

after CAS intake. In line with this, serum concentrations of  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate were lower and fasting and postprandial NEFA concentrations were markedly reduced after 8 weeks CAS supplementation, especially when compared to the SAL group. Reduced fat mobilisation through insulin suppression may also explain the lower NEFA concentration observed in this group.

Adipokines play an important role in insulin resistance and cardiovascular complications associated with obesity [41]. Obese patients with insulin resistance are characterised by low circulating adiponectin concentration, and insulin resistance and increased fat mass is associated with increased circulating concentration of leptin [41]. The increased fasting concentration of adiponectin with no change in leptin in the COD group is interesting since the postprandial glucose concentration also was improved when considering within-group changes, although no differences were seen for these parameters when the experimental

groups were compared, thus supporting the assumption that adiponectin may act as an insulin sensitizer and improve insulin sensitivity [42, 43] in this group. Increased adiponectin concentration in COD group is in agreement with studies reporting increased adiponectin concentrations after fat fish intake in non-obese adults [44, 45], and suggest that not only n-3 long chain PUFAs [46–48], but also fish protein may be capable of increasing circulating adiponectin concentrations in humans. Conflicting results are published on the effect of fish intake on leptin concentration in humans, showing no effects on leptin concentration after salmon intake [44], whereas a high consumption of freshwater fish is associated with low leptin concentrations [49]. Fasting leptin concentration was increased solely in the CAS group, which was surprising in light of the improved postprandial glucose regulation observed in the CAS group. Adiponectin concentration was not affected in HER, SAL and CAS groups, but the increased adiponectin concentration in COD group

**Table 7** Plasma fasting concentrations of leptin, adiponectin and NEFA (medians and 25th, 75th percentiles)

Intervention groups	Baseline		8 weeks		$P^{\dagger}$	$P^{\ddagger}$	$P^{\S}$
	Median	25th, 75th percentile	Median	25th, 75th percentile			
Fasting leptin (ng/ml)							
HER	17.0	8.9, 26.3	19.8	7.6, 26.0	0.32	0.28	0.73 <sup>A</sup>
SAL	17.8	7.4, 28.0	18.8	6.6, 26.8	0.42	0.14	0.44 <sup>B</sup>
COD	20.6	8.4, 29.9	17.7	9.2, 27.5	0.93	0.042	0.66 <sup>C</sup>
CAS	20.6	14.9, 30.0	22.9	14.3, 34.2	0.0094		
Fasting adiponectin (ug/ml)							
HER	12.5	9.1, 14.6	13.2	9.6, 17.5	0.097	0.89	0.21 <sup>A</sup>
SAL	11.4	8.5, 17.8	11.0	7.6, 18.1	0.77	0.13	0.44 <sup>B</sup>
COD	13.2	11.3, 20.5	16.2	10.6, 23.3	0.041	0.44	0.072 <sup>C</sup>
CAS	10.2	8.8, 15.5	10.8	10.8, 15.7	0.20		
Fasting NEFA (mmol/l)							
HER	0.68	0.51, 0.86	0.61	0.51, 0.74	0.067	0.047	0.25 <sup>A</sup>
SAL	0.56	0.49, 0.65	0.61	0.45, 0.69	1.00	0.0072	0.50 <sup>B</sup>
COD	0.60	0.48, 0.75	0.60	0.46, 0.67	0.44	0.013	0.55 <sup>C</sup>
CAS	0.62	0.54, 0.76	0.50	0.34, 0.62	0.00019		

No differences were observed between the groups at baseline (Kruskal–Wallis test). Results are presented for twenty participants in the HER group, twenty participants in the SAL group, eighteen participants in the COD group and nineteen participants in the CAS group

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture, NEFA non-esterified fatty acids

<sup>†</sup>Within-group changes are tested using Wilcoxon's signed-rank test

<sup>‡</sup>Changes within fish protein groups are compared with the CAS group using the Mann–Whitney test

<sup>§</sup>Changes within fish protein groups are compared using the Mann–Whitney test

<sup>A</sup>HER group is compared to SAL group

<sup>B</sup>HER group is compared to COD group

<sup>C</sup>SAL group is compared to COD group

is of great interest as alteration in adiponectin is found to precede changes in insulin resistance [50].

Plasma triacylglycerol concentration has been shown to be higher after cod intake compared to whey protein intake after a fat-rich meal [26], however, animal studies have shown beneficial effects of fish proteins on regulation of triacylglycerols and cholesterol concentrations [20, 21, 23, 51, 52] as well as fatty acid metabolism [22, 23]. We have previously shown that supplementation with cod protein (6 g/day) reduced LDL-cholesterol in healthy overweight adults with no effect on serum concentrations of triacylglycerols, total cholesterol and HDL-cholesterol [24, 53]. Similar effects have been seen in studies with milk proteins, where whey intake lowered circulating triacylglycerol concentration but showed no effects on total, HDL or LDL cholesterol when compared to casein intake [54], whereas an acute study in patients with metabolic syndrome found no effect on whey intake on triacylglycerol concentration after a fat-rich meal [27]. The triacylglycerol-lowering effect of long chain n-3 PUFAs from fish is well documented, but the effects on cholesterol metabolism are still debated [55–60]. In the present study, long-chain n-3 PUFAs were found only in cod protein, with daily intake of 45 mg from tablets, and we observed a

marginal decrease in n-3/n-6 PUFA ratio in HER group, with no change in n-3 and n-6 PUFAs in the other groups. This is in contrast to our observations in rat studies using proteins from the same production batches where rats fed proteins from herring or cod had higher serum ratio of n-3 to n-6 PUFA compared to rats fed a casein–whey mixture [22, 23]. Supplementation of protein from fish or milk did not affect the serum concentrations of triacylglycerol, total cholesterol or LDL and HDL cholesterol in the present study, and it is possible the dose may have been too low to affect serum fatty acids and lipids.

The protein intake from the supplements in this study was very low relative to the average protein intake for all participants at both visits, which was estimated to be 92 g/day, and consequently the 2.5 g of protein from the supplements accounted for only 2.7% of the daily total protein intake. It is unlikely that the intake of individual amino acids in such small amounts from the supplements would be sufficient to induce any effects on metabolism. Motifs with glucose lowering capacities have been identified in various species of fish including cod as well as in milk [61–63], and we, therefore, speculate that an improvement in postprandial glucose regulation after COD and CAS intake could be linked to the

**Table 8** Fatty acids in fasting serum samples (medians and 25th, 75th percentiles)

Intervention groups	Baseline		8 weeks		$P^{\dagger}$	$P^{\ddagger}$	$P^{\S}$
	Median	25th, 75th percentile	Median	25th, 75th percentile			
Total SFA (g/100 g fatty acids)							
HER	33.9	31.7, 34.6	34.0	32.4, 35.0	0.031	0.19	0.99 <sup>A</sup>
SAL	33.4	32.1, 35.2	33.7	33.0, 35.5	0.039	0.13	0.28 <sup>B</sup>
COD	33.2	32.5, 34.2	33.9	32.0, 34.7	0.91	0.91	0.16 <sup>C</sup>
CAS	33.8	33.1, 34.5	33.9	32.6, 35.2	0.78		
Total MUFA (g/100 g fatty acids)							
HER	29.0	28.1, 30.1	28.2	26.7, 30.7	0.87	0.14	0.74 <sup>A</sup>
SAL	29.3	16.5, 32.3	28.3	26.5, 30.6	0.41	0.28	0.85 <sup>B</sup>
COD	27.6	25.4, 29.6	28.6	25.8, 29.5	0.87	0.076	0.67 <sup>C</sup>
CAS	28.9	27.7, 30.1	26.7	25.0, 28.7	0.020		
Total n-3 PUFA (g/100 g fatty acids)							
HER	3.6	3.0, 4.6	3.3	2.9, 4.0	0.0042	0.0062	0.44 <sup>A</sup>
SAL	3.3	3.2, 5.0	3.5	3.1, 4.2	0.25	0.19	0.21 <sup>B</sup>
COD	3.9	3.1, 4.4	3.5	3.1, 4.2	0.76	0.56	0.40 <sup>C</sup>
CAS	3.7	3.1, 4.1	3.6	3.3, 4.3	0.31		
Total n-6 PUFA, g/100 g fatty acids							
HER	33.4	31.9, 36.1	33.9	31.5, 36.7	0.80	0.16	0.72 <sup>A</sup>
SAL	33.0	30.3, 35.8	33.9	32.4, 35.4	0.65	0.40	0.82 <sup>B</sup>
COD	35.3	32.2, 37.1	34.2	32.6, 37.4	0.98	0.22	0.74 <sup>C</sup>
CAS	33.9	32.0, 37.2	35.7	31.6, 37.3	0.061		
Ratio n-3/n-6 PUFA							
HER	0.10	0.09, 0.14	0.10	0.09, 0.11	0.049	0.36	0.99 <sup>A</sup>
SAL	0.11	0.10, 0.14	0.10	0.09, 0.13	0.076	0.27	0.13 <sup>B</sup>
COD	0.11	0.09, 0.12	0.10	0.09, 0.13	0.98	0.58	0.19 <sup>C</sup>
CAS	0.10	0.09, 0.12	0.10	0.09, 0.12	0.46		

No differences were observed between the groups at baseline (Kruskal–Wallis test). Results are presented for seventeen participants in the HER group, seventeen participants in the SAL group, seventeen participants in the COD group and fifteen participants in the CAS group

HER herring hydrolysate, SAL salmon hydrolysate, COD cod protein, CAS casein–whey mixture

<sup>†</sup>Within-group changes are tested using Wilcoxon's signed-rank test

<sup>‡</sup>Changes within fish protein groups are compared with the CAS group using the Mann–Whitney test

<sup>§</sup>Changes within fish protein groups are compared using the Mann–Whitney test

<sup>A</sup>HER group is compared to SAL group

<sup>B</sup>HER group is compared to COD group

<sup>C</sup>SAL group is compared to COD group

presence of bioactive motifs with antidiabetic effects. When searching for bioactive peptide sequences in the fish protein hydrolysates, the antidiabetic motifs GPL, IPI and VW were found in both herring and salmon hydrolysates, whereas the antidiabetic motifs GPAE [42] and LPGP [43] were found only in the herring hydrolysate [23]. Partially in line with this, HER, but not SAL, reduced serum concentrations of  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate, thus indicating improved insulin sensitivity. We were surprised to find no effects on serum cholesterol in HER and SAL groups, since we previously have identified hypocholesterolemic motifs in both the herring and the salmon hydrolysates [23]. However, although bioactive motifs have

been identified in both fish and milk proteins, it is uncertain whether these motifs are in the form of small detached peptides that can be absorbed directly from the intestine without further proteolysis, or whether these motifs are released as bioactive peptides after intestinal proteolysis. Again, the low amounts of fish protein consumed may not be sufficient to reveal the potential antidiabetic and hypocholesterolemic properties of these supplements.

Physical activity is known to improve glucose regulation and insulin sensitivity [64]; however, the level of physical activity was not changed within the groups from baseline to endpoint. In addition, no changes were seen in body composition and energy, protein, carbohydrate and total fat intake

within the groups during the study period, therefore, any changes in glucose regulation observed are most likely direct effects of the protein supplements and not an indirect consequence of lifestyle modification. An improved postprandial glucose regulation in COD and CAS groups may have been caused by enhanced postprandial insulin sensitivity, rather than increased insulin secretion, since the relative increase from fasting to postprandial insulin c-peptide concentration was unchanged in COD group and reduced in CAS group. This was supported by findings of reduced serum concentrations of  $\beta$ -hydroxybutyrate and fructosamine in COD group and reduced serum  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate concentrations in CAS group. The reduced serum  $\alpha$ -hydroxybutyrate, acetoacetate and  $\beta$ -hydroxybutyrate concentrations after HER intake also may indicate improved insulin sensitivity although no changes in postprandial glucose, insulin and insulin c-peptide concentrations were observed, while SAL did not affect any of the measured markers of glucose regulation and insulin sensitivity.

There are some limitations to this study. The sample size was not calculated in advance, since to our knowledge this is the first clinical study to investigate a low dose of fish proteins (2.5 g/day). Therefore, the present study is considered to be a pilot study and enables sample size calculations in future studies with similar designs. We cannot rule out the possibility that the study was too small to identify minor effects of fish and milk proteins on glucose regulation and lipid metabolism. Another weakness of the present study is that the intervention groups did not ingest an equal number of tablets due to practical problems with packing of the tablets. The herring and salmon protein hydrolysates were hygroscopic, and therefore, more of the stabilization and filling agents had to be blended in before the mixture could be compressed into tablets with the same technical quality and hardness as the COD and CAS tablets, resulting in lower protein content in HER and SAL tablets. This may have had a more pronounced effect on participant compliance in HER and SAL groups relative to the COD and CAS groups. In the present pilot study we did not include a non-protein intervention (control) group, however, such group will be of great value for direct comparisons with the intervention proteins from fish and milk and will be included in the main study.

To conclude, the results from this relatively small pilot study suggest that a daily intake of 2.5 g of proteins from fish or milk may be sufficient to improve glucose regulation but did not affect circulating lipid concentrations in overweight adults. The effects were most pronounced after supplementation with proteins from cod, herring and milk, whereas salmon protein did not affect any of the measurements related to glucose regulation in the present study. The results from this pilot are promising, especially with regard to effects on glucose regulation, however, a larger study

should be conducted to clarify whether a daily dose of 2.5g of protein from fish or milk is sufficient to affect glucose regulation and lipid metabolism in a healthy population with obesity or overweight.

**Author contributions** GM, TR, IH and OAG formulated the research question and designed the study. RS, AC, TR and IH prepared the fish protein hydrolysates for the study. IHH, ISL, OS, KHW and OAG conducted the clinical study. SAM, KAB, AM, PMU and OAG analysed the data and performed statistical analyses. OAG drafted the paper and had primary responsibility for the final content. All authors have contributed to the writing and approved the final manuscript. We thank all participants who have contributed to the current study.

**Funding** The present research has been supported by funding from the Bergen Medical Research Foundation and the Regional research fund of Western Norway (Project No. 212625). These sponsors were not involved in the design of the study, data collection, analysis and interpretation of data, writing of the article or in the decision to submit the article for publication.

## Compliance with ethical standards

**Conflict of interest** TR was during the time this study was planned and conducted employed at Nutrimar AS, a company owned by the investment company Kverva AS who also own SalMar ASA. SalMar ASA is one of the world's largest producers of farmed salmon, and provided fresh salmon backbones for this study. IH is CEO and Chairman of Blue Protein, a company that commercialises new products based on fish proteins from fish by-products. Nutrimar AS, Kverva AS, SalMar ASA and Blue Protein were not involved in on-site data collection. The other authors declare no conflicts of interest.

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## Affiliations

Ida Heir Hovland<sup>1</sup> · Ingrid Sande Leikanger<sup>1</sup> · Oddbjørg Stokkeland<sup>1</sup> · Kaia Hevrøy Waage<sup>1</sup> · Svein A. Mjøs<sup>2,3</sup> · Karl A. Brokstad<sup>4</sup> · Adrian McCann<sup>5</sup> · Per Magne Ueland<sup>6,7</sup> · Rasa Slizyte<sup>8</sup> · Ana Carvajal<sup>8</sup> · Gunnar Mellgren<sup>6,9</sup> · Tore Remman<sup>10</sup> · Ingmar Høgøy<sup>11</sup> · Oddrun A. Gudbrandsen<sup>1,12</sup> 

<sup>1</sup> Dietary Protein Research Group, Department of Clinical Medicine, University of Bergen, 5020 Bergen, Norway

<sup>2</sup> Department of Chemistry, University of Bergen, PO Box 7803, 5020 Bergen, Norway

<sup>3</sup> Nofima BioLab, Oasen, PO Box 1425, 5828 Bergen, Norway

<sup>4</sup> Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Bergen, Norway

<sup>5</sup> Bevitall AS, Jonas Lies veg 87, 5021 Bergen, Norway

<sup>6</sup> Department of Clinical Science, University of Bergen, 5021 Bergen, Norway

<sup>7</sup> Laboratory of Clinical Biochemistry, Haukeland University Hospital, 5021 Bergen, Norway

<sup>8</sup> SINTEF Ocean, Brattørkaia 17 C, 7010 Trondheim, Norway

<sup>9</sup> Hormone Laboratory, Haukeland University Hospital, Bergen, 5021 Bergen, Norway

<sup>10</sup> Nutrimar AS, Industrivei 23, 7266 Kverva, Norway

<sup>11</sup> Blue Protein, 5393 Storebø, Norway

<sup>12</sup> Department of Clinical Medicine, University of Bergen, Haukeland University Hospital, 5021 Bergen, Norway