No effect of salmon fish protein on 2h-glucose in adults with increased risk of type 2 diabetes: a randomised controlled trial

K.S. Hustad¹, I. Ottestad^{1, 2}, M. Hjorth¹, K.T. Dalen¹, T. Sæther³, N.A. Sheikh¹, M. Strømnes¹, S.M. Ulven¹, K.B. Holven^{1, 4}

¹ Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway

² The Clinical Nutrition Outpatient clinic, Section of Clinical Nutrition, Department of Clinical Service, Division of Cancer Medicine, Oslo University Hospital, Norway

³ Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Norway

⁴ National Advisory Unit on Familial Hypercholesterolemia, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, Norway

Corresponding author:

Kirsten B. Holven, Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, P.O Box 1046 Blindern, 0317 Oslo, Norway

Telephone: +47 22 85 13 61, e-mail: kirsten.holven@medisin.uio.no

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18 **Abstract**

19 The association between fish consumption and decreased risk of CVD is well-documented.
20 However, studies on health effects of fish consumption, suggest that other components than
21 omega-3 polyunsaturated fatty acids have beneficial cardiometabolic effects, including effects on
22 glucose metabolism. The aim of the present study was to investigate effects of salmon fish
23 protein on cardiometabolic risk markers in a double-blind, randomised controlled parallel trial.
24 We hypothesised that daily intake of a salmon fish protein supplement for eight weeks would
25 improve glucose tolerance in persons with increased risk of type 2 diabetes mellitus. Our primary
26 outcome measure was serum glucose (s-glucose) two hours after a standardized oral glucose
27 tolerance test (OGTT). In total, 88 adults with elevated s-glucose levels were randomised to 7.5 g
28 salmon fish protein/day or placebo, and 74 participants were included in the analysis. We found
29 no significant effect of salmon fish protein supplementation on our primary outcome or other
30 markers related to glucose tolerance, serum lipids, weight or blood pressure compared to
31 placebo. The present study does not support the hypothesis that daily intake of a salmon fish
32 protein supplement for eight weeks improves glucose tolerance in persons with increased risk of
33 type 2 diabetes mellitus. The study was registered at ClinicalTrials.gov (ID: NCT03764423).

Introduction

Diabetes mellitus type 2 (T2DM) is a considerable contributor to the global burden of disease ⁽¹⁾. In 2019, worldwide prevalence of T2DM among adults was estimated to 8.4 %, where only half being diagnosed. In addition, 7.5 % of the adult population were estimated to have impaired glucose tolerance ⁽²⁾.

There is a strong correlation between diabetes and cardiovascular disease (CVD) ⁽³⁾, which is the number one cause of death globally ⁽⁴⁾ and a major cause of mortality in people with diabetes ⁽⁵⁾. Adults with diabetes has a two-three times increased risk of CVD, and CVD events generally occur at an earlier age in people with diabetes than people without diabetes ⁽⁵⁾. A healthy diet is important to prevent CVD and T2DM ⁽⁶⁾ and the association between fish consumption and decreased risk of CVD is well-documented ⁽⁷⁻⁹⁾. The beneficial effects of fish consumption have largely been attributed to marine omega-3 polyunsaturated fatty acids (n-3 PUFAs) present in fatty fish ⁽¹⁰⁾. However, studies on health effects of lean fish consumption, suggest that other components than n-3 PUFAs have beneficial cardiometabolic effects ⁽¹¹⁻¹⁴⁾.

Both lean and fatty fish contain other potential health-promoting components such as taurine, vitamin D, vitamin B12, iodine, selenium ⁽¹⁵⁾ and more unspecified components such as bioactive peptides ⁽¹⁶⁾.

Both lean and fatty fish contain other potential health-promoting components such as taurine, vitamin D, vitamin B12, iodine and selenium ⁽¹⁵⁾. Additionally, peptides with a specific amino acid sequence, and with known bioactivity, have been isolated from by-products from lean and fatty fish ^(17, 18). *In vitro* and animal studies have suggested that fish protein have beneficial effects on e.g. cardiometabolic markers, including markers related to blood glucose metabolism ^(16, 19-21). Fish protein peptides are formed during digestion or from enzymatically treatment, and it has been hypothesized that peptides may act locally in the gut or peripherally ^(16, 21). Animal studies have shown improved postprandial glucose regulation, albeit higher weight gain ⁽²¹⁾, resistance to high-fat-diet-induced obesity ^(22, 23), and reduced plasma lipids such as triglycerides and total cholesterol ^(22, 24) when fed a diet rich in hydrolysed salmon protein. Clinical trials with

fish protein given as supplements, mainly from lean fish, have suggested beneficial cardiometabolic effects ⁽²⁵⁻³⁰⁾, including improved glucose metabolism ⁽²⁹⁾. However, clinical trials in humans with protein supplements derived from fatty fish are sparse, and the studies are small and with inconsistent results on cardiometabolic risk markers ^(26, 27, 31-33).

The overall aim of the FishMeal human intervention study was to investigate effects of salmon fish protein on cardiometabolic risk markers. We hypothesized that daily intake of a salmon fish protein supplement for eight weeks would improve glucose tolerance in persons with increased risk of T2DM. Our primary outcome was changes from baseline in serum glucose (s-glucose) measured after a two-hour oral glucose tolerance test (2h-OGTT). Secondary outcomes were changes from baseline in other markers related to glucose tolerance: fasting s-glucose, fasting s-insulin, 2h-OGTT-s-insulin, HOMA-IR and HbA1c. Other pre-specified outcomes were changes from baseline in body weight and markers related to lipid metabolism: triglycerides (TG) and total-, LDL-, and HDL-cholesterol.

Methods

Participants

The study was conducted at the University of Oslo, Norway from August 2018 until September 2019. We recruited participants through advertisements in social media and medical practices at the University of Oslo. The text in the advertisement was directed at people at risk of T2DM. After a telephone interview, we invited eligible participants to a screening visit to further check eligibility criteria. Inclusion criteria were age ≥ 20 years and elevated blood glucose defined as; either fasting s-glucose ≥ 5.6 mmol/l, 2h-OGTT-s-glucose ≥ 6.5 mmol/l or HbA1c ≥ 40 mmol/mol (≥ 5.8 %). Exclusion criteria were diabetes (defined as fasting s-glucose ≥ 7.0 mmol/l, 2h-OGTT-s-glucose ≥ 11.1 mmol/l or HbA1c ≥ 40 mmol/mol (≥ 5.8 %)), high fish/seafood intake (>450 g/week), fish or shellfish allergy and age-related elevated blood pressure (≥ 70 years: $\geq 180/110$ mmHg, $>40-70$ years: $\geq 170/100$ mmHg and ≤ 40 years: $\geq 160/100$ mmHg). Further exclusion criteria were use of prescription drugs related to diabetes, inflammation or systemic use of corticosteroids, or unstable use (defined as change of dose during the last three months) of lipid

lowering drugs, thyroxine, blood pressure lowering drugs and drugs affecting appetite. In addition, we excluded participants with unstable use (defined as change of dose during the last month) of dietary supplements including n-3 PUFAs, daily use of protein supplement powder, and participants who were pregnant, breastfeeding or planning pregnancy. Furthermore, all participants had to have a stable body weight (defined as $\pm 5\%$) during the last three months and not be planning changes in body weight during the intervention period.

Ethics

The study was conducted according to the guidelines laid down in the Declaration of Helsinki. All participants gave their written informed consent, and the Regional Ethics Committee for Medical Research in South East Norway approved the study. The study was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT03764423). The postprandial “Fish protein Ex Vivo” study, assessing uptake of the study product, was registered as a separate study (ClinicalTrials.gov Identifier: NCT04078958).

Study design

We conducted an 8-week double blind, randomised controlled parallel study. Before the baseline visit, all participants performed a 2-4-week washout period where intake of fish and seafood were reduced to a maximum of one serving (150 g) per week. We instructed participants in both groups to consume 10 capsules together with a meal three times per day for 8 weeks (in total 30 capsules per day). All participants were advised to maintain their usual lifestyle habits throughout the study, without changing their physical activity and dietary habits including supplement use, except for a reduction in fish and seafood intake to a maximum of one serving (150 g) per week. Clinical and blood laboratory assessments were performed at baseline and after 8 weeks of follow-up. In addition, the participants came to the study centre after 4 weeks of follow-up to receive more of the study product. We sent a text message 2–3 days before all visits as a reminder of how to prepare for the upcoming visit.

Blinding and randomisation

Participants were stratified by sex (male, female) and age (<50 y, ≥50 y) prior to a block randomisation with use of an external statistician. (Health Services Research Unit (HØKH), Akershus University Hospital, Lørenskog, Norway; and Faculty Division Akershus University Hospital, University of Oslo, Blindern, Oslo, Norway). The randomisation allocations, selected consecutively, were sent to the product packaging personnel on demand, according to strata information of newly recruited participants.

To ease the management for the participants, capsules were packed in blister sheets (30 capsules per sheet) and delivered in boxes (7 sheets per box). Boxes (fish protein and placebo) were identical in appearance, and were only identifiable by numbers on the containers. The study was double-blinded, as neither the participants, the study investigator collecting data nor the outcome adjudicators knew which group the participants were assigned to. The randomisation code was concealed from the study investigators until the statistical analyses were completed.

Study product

The experimental group received capsules containing salmon fish protein (250 mg/capsule), microcrystalline cellulose (240 mg/capsule), antioxidants (tocopherols and rosemary extract) and excipients (magnesiumstearate; 5 mg/capsule, tricalciumphosphate; 5 mg/capsule and silisumdioxide; 2.5 mg/capsule). The placebo group received capsules containing microcrystalline cellulose (250 mg/capsule) and antioxidants and excipients similar to the fish protein capsules, but without amino acids. The salmon fish protein contained 69.7 g protein and 13.2 g fat/100 g. Table 1 shows the amino acid composition and main groups of fatty acids of the salmon fish protein used in this study. In the fish protein group, the daily dosage of capsules provided 7.5 g of salmon fish protein, corresponding to a total of 5.2 grams of salmon protein. Mowi ASA (Bergen, Norway) supplied the salmon fish protein and Optipharma AS (Drøbak, Norway) produced the capsules in transparent bovine gelatine capsule shells (96 mg gelatine/capsule) (ACG Europe Ltd., Reading, UK). Before and after encapsulation, the fish protein and capsules were stored at 5.5 °C, and participants were instructed to store the capsule containers at 4 °C during the intervention period. Before encapsulation, and regularly during the

intervention period, the content of unwanted microorganisms (histamine, aerobic microorganisms, E.coli, salmonella) were analysed in the fish protein. Before encapsulation, we also analysed the content of contaminants (Eurofins Food & Feed Testing Norway AS, Moss, Norway). We did not detect any increase in unwanted microorganisms in the fish protein during the intervention.

Uptake of study product

To investigate whether fish protein was taken up into the circulation, we performed a postprandial analysis of serum amino acids 1h after intake in five healthy participants. In short, five healthy, male participants were recruited from the University of Oslo from October to November 2019. Inclusion criteria were age > 20 years and BMI between 18.5 - 24.9 kg/m². Exclusion criteria were known diabetes, elevated blood pressure, pregnancy, breastfeeding or allergy/intolerance to fish. Participants arrived fasting on the morning of the postprandial test, and consumed 30 capsules containing a total of 5.2 g salmon protein (the same amount as the daily dose in the present RCT) with 0.5 l water. Blood samples were taken at fasting and 60 minutes after capsule intake. Participants were not allowed to consume dietary supplements or fish the day before the test.

Serum amino acid concentrations were measured by high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS), as previously described⁽³⁴⁾. Chromatographic separation was performed on a Phenomenex Kinetex Core Shell C18 system (100 x 4.6 mm, 2.6 µm), with an aqueous solution of formic acid (0.5 %) and heptafluorobutyric acid (0.3 %), and acetonitrile. Linear calibration curves of the peak area ratios of analytes and internal standards were used for quantification.

Compliance

The participants received boxes with capsules at baseline and after 4 weeks of follow-up, and were instructed to deliver all blister sheets, both empty and full, at the end-of-study visit. Compliance was assessed by capsule count. The number of capsules consumed during the intervention period were counted, and divided by the number of capsules scheduled for the

intervention period ⁽³⁵⁾. Participants with compliance less than 70 % would be excluded from the analysis.

Blood sampling and standard laboratory analysis

Participants were instructed to avoid consumption of alcohol and doing vigorous physical activity the day before blood sampling. Venous blood samples were drawn after an overnight fast (≥ 10 h). Serum was obtained from silica gel tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and kept at room temperature for >30 - 60 min, until centrifugation ($1500 \times g$, 15 min). Plasma was obtained from K₂EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), immediately placed on ice, and centrifuged within 10 min ($2000 \times g$, 4°C, 15 min). Lithium-Heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and K₂EDTA tubes with whole blood were kept at room temperature. Serum and plasma concentrations of fasting glucose, insulin, HbA1c, TG, total-cholesterol, LDL-C, HDL-C, hsCRP, creatinine, eGFR, ASAT, ALAT, Gamma-GT and mercury, and glucose and insulin after a 2h-OGTT, were measured by standard methods at an accredited routine laboratory (Først Medical Laboratory, Oslo, Norway).

Oral glucose tolerance test

An OGTT was conducted at baseline and at the end-of-study visit. Venous blood samples were drawn, and within 10 minutes, the participants were instructed to drink a 75-g anhydrous glucose drink (Esteriplas, Feira, Portugal) in less than 5 minutes. Participants were instructed to remain fasting, remain in the waiting room and refrain from any activity until the postprandial blood samples were drawn 120 minutes after finishing the glucose drink.

Clinical assessment

We measured body weight on a digital scale (Seca GmbH, Hamburg, Germany) in light clothing without shoes and height with a stadiometer (Seca GmbH, Hamburg, Germany). Blood pressure was measured by a Carecape V100 monitor (GE Healthcare, Chicago, IL, USA) in a sitting

position, on the non-dominant arm after a 10 min rest. We obtained three measurements with a one-minute interval, and calculated the average value of the last two measurements.

Dietary assessment

Habitual dietary intake was assessed prior to the intervention through a semi-quantitative food frequency questionnaire (FFQ) designed to capture dietary habits during the last year⁽³⁶⁾. The FFQ included questions about intake of 270 food items, including six questions about cold cuts and spreads made of fish and 12 questions about fish eaten for dinner. The options for frequency of consumption ranged from several times per day to once a month, with options for portion sizes based on household units such as slices, pieces and spoons. The same FFQ was used to assess the participants' diets during the 8-week intervention.

Statistical analysis

Power calculations estimated that 120 participants (including a 20 % dropout rate) were required to obtain 80 % power with a type I error of 5 % to detect a clinically relevant difference between the two groups of 0.4 mmol/l (standard deviation (SD) 0.7) in changes from baseline in 2h-OGTT-s-glucose. Descriptive data are presented as mean (SD) or median (quartiles, Q1-Q3) for continuous variables or as frequency (percentage, %) for categorical variables. We used paired t-tests to evaluate differences in energy and nutrient intake between the groups. Differences between the groups in primary, secondary and other pre-specified outcomes were tested with a linear regression model (outcome variable ~ intervention group + outcome variable at baseline), hereafter called crude model. We performed the same analysis adjusting for strata (age, sex) and weight change in addition to the outcome variable at baseline (outcome variable ~ intervention group + outcome variable at baseline + age + sex + weight change), hereafter called the adjusted model. Skewed variables (fasting s-insulin, 2h-OGTT-s-insulin, HOMA-IR, TG and weight) were log-transformed before analysis. Results from the regression analysis are presented as B-coefficient with 95 % confidence interval (CI) or logB-coefficient with 95 % CI for skewed variables. $P < 0.05$ was considered significant. The models were checked for independence and

normality of the residuals. Statistical analysis were performed in Stata/MP 16.1 (StataCorp LLC, College Station, TX, USA) ⁽³⁷⁾.

Results

In total 717 participants were assessed for eligibility, 88 were randomly assigned, 83 received allocated interventions and seven were lost to follow-up. Thus, 76 participants completed the study. All participants had a capsule compliance >70 %. Two participants were non-compliant with the approved study protocol and were not included in the statistical analysis (Fig. 1). Baseline characteristics of the 74 participants included in the present study are shown in table 2. The participants were 56 years of age (Q1-Q3: 48-64), with a mean BMI of 33.5 ± 4.7 kg/m², and 64 % were female.

Primary and secondary outcomes

At baseline, fasting s-glucose was 5.4 ± 0.5 mmol/L in the fish protein group and 5.7 ± 0.6 mmol/L in the placebo group, and 2h-OGTT-s-glucose was 5.8 ± 1.4 mmol/L in the fish protein group and 6.3 ± 1.5 mmol/L in the placebo group. During the intervention period, we found no statistically significant differences on fasting s-glucose, 2h-OGTT-s-glucose, fasting s-insulin, HOMA-IR or HbA1c, whereas 2h-OGTT-s-insulin was significantly increased in the crude model (logB 0.23 (95 % CI: 0.01, 0.45), $P < 0.05$). Results on primary and secondary outcomes using both the crude and the adjusted model are shown in table 3.

Other pre-specified outcomes

During the intervention, no significant difference were found for total-cholesterol, LDL-C, HDL-C and triglycerides between the groups (table 4). Median weight increase was 1.0 kg (Q1-Q3: -0.2-2.0) in the fish protein group and 0.4 kg (Q1-Q3: -0.8-1.3) in the placebo group ($P = 0.08$).

Energy and macronutrient intake

At baseline, median daily energy intake was 9295 kJ/d (Q1-Q3: 7931-11760) in the fish protein group and 9257 kJ/d (Q1-Q3: 7931-10618) in the placebo group. There were no significant

changes in the macronutrient, sugar, fibre and energy intake between the groups during the intervention period (table 5). The fish protein group reported a reduction in energy intake of 559 kJ/d (Q1-Q3: -1278-462) and the placebo group reported a reduction of 971 kJ/d (Q1-Q3: -2828-417) ($P = 0.24$). Contribution of energy and macronutrients from the study products are not included in the analysis of dietary data.

Systolic and diastolic blood pressure

We also measured systolic (SBP) and diastolic blood pressure (DBP). In both groups, 31 % of the participants used blood pressure lowering drugs (table 2). At baseline, SBP was 119 ± 13 mmHg and DBP 71 ± 10 mmHg in the fish protein group and 122 ± 16 mmHg and 71 ± 10 mmHg in the placebo group. During the intervention period, there were no significant changes in SBP ($P = 0.45$) or DBP ($P = 0.79$) between the groups (table 4).

Uptake of study product

Postprandial analysis of serum amino acids was performed 1h after intake in five healthy participants. A non-significant increase in plasma levels of most amino acids were seen (supplementary table 1 and supplementary figure 1).

Discussion

In the present study, we investigated the effects of a daily intake of salmon fish protein on several cardiometabolic risk markers among adults with increased risk of T2DM. We found no beneficial effect of salmon fish protein supplementation on markers related to glucose tolerance, serum lipids, weight or blood pressure compared to the placebo group. The present study does not support the hypothesis that daily intake of a salmon fish protein supplement (7.5 g/d) for eight weeks improves glucose tolerance in persons with increased risk of T2DM.

To the best of our knowledge, this is the first clinical trial exploring the health effect of a fatty fish protein supplement in adults with elevated blood glucose levels.

Few clinical trials, of which three were RCTs ongoing for 6-12 weeks, have investigated health effects of protein supplements from fatty fish ^(26, 31, 32). In line with the results in the present study, no between-group differences in markers related to glucose regulation or lipid metabolism was observed in overweight adults (n=77) assigned to 2.5 g protein/day (8 weeks) from either herring, salmon, cod or casein/whey, except from lower glucose area under the curve in the casein/whey group than the salmon group ⁽³¹⁾. Nor did Nenseter *et al* observe improvement in risk factors for coronary heart disease in adults with hypercholesterolemia (n=70) from 10 g fish powder/day (12 weeks) from herring in patients following the National Cholesterol Education Program Step I Diet ⁽³²⁾. In contrast, an RCT on lean fish protein in overweight adults (n=34) assigned to a cod protein supplement (3 g/day for 4 weeks and 6 g/day for 4 weeks) demonstrated lower fasting glucose and glucose, insulin and C-peptide after a 2h-OGTT in the cod supplement group than the placebo group ⁽²⁹⁾. However, these findings were not supported in a later RCT conducted by the same research group in overweight or obese adults (n=42) consuming 6 g protein/day (8 weeks) from cod residuals ⁽³⁸⁾. An RCT in overweight adults (n=110) on 1.4 or 2.8 g/day (90 days) of blue whiting protein hydrolysate given as part of a food supplement, significantly improved body composition, decreased body weight, and increased cholecystokinin and glucagon like peptide-1 compared to whey protein ⁽²⁸⁾. Both doses provided equal results. None of the RCTs using fish protein supplements have found between-group effects on markers related to glucose tolerance. However, investigating the acute effect of fish protein supplementation, a double-blind crossover postprandial trial in healthy participants (n=41) found that 20 mg cod protein hydrolysate/kg bodyweight consumed before a standardized breakfast meal reduced postprandial insulin concentrations, without affecting blood glucose, compared to casein ⁽²⁵⁾. In addition to the use of different protein doses between the studies, different fish species have different amino acid composition that may explain the inconsistent results. Including large amounts of fish in the diet, between-group effects are reported on insulin sensitivity and postprandial C-peptide in studies with lean fish ^(11, 12). Salmon consumed as whole fillets (750 g/week), have shown improved postprandial glucose response and less increase in C-peptide response in an RCT in overweight or obese adults (n=65) compared to cod fillet (8 weeks) ⁽³⁹⁾. In the present study, participants reported a protein intake of about 93 g/day (results not shown). In addition, the fish protein capsules provided 5.2 g protein/day for participants in

the fish protein group, the same protein amount as in approx. 25 g salmon fillet (175 g/week). These results suggest that the daily dose of fish protein provided in the present study may be too low to detect an effect, and indicates that fish protein may have to be consumed in larger amounts than what a supplement can provide.

In the present study, the participants in the fish protein group had a non-significant weight gain and less reduction in reported energy intake during the intervention period compared to the placebo group. In contrast, Framroze *et al* found that 16 g of salmon protein hydrolysate/day consumed together with breakfast, in a 6-week RCT in overweight participants (n=48), reduced BMI by 5.6 % compared to whey protein isolate, which did not affect weight ⁽²⁶⁾. In addition, similar to the use of different fish species and protein doses, different protein sources used as control diets makes it difficult to compare results. Most intervention studies have compared lean fish with fatty fish or a non-seafood diet containing equal amounts of protein from lean meat, poultry, eggs and dairy products.

Although daily intake of a salmon fish protein supplement did not improve the cardiometabolic risk markers we investigated in this study, we did not detect any harmful effects of the supplement. The High Level Panel of Experts on Food Security and Nutrition have presented utilization of by-products as one of the solutions to reduce food losses and waste ⁽⁴⁰⁾. Thus, the potential for fish by-products utilized for human consumption should be further investigated, e.g. adding fish protein to food products.

Norway is one of the world's largest aquaculture and fishing nations. In 2018, 27 % of all catch from the fishery and aquaculture industry ended as by-products, mainly utilized for animal feed production ⁽⁴¹⁾. Only 13 % of the by-products are used for human consumption ⁽⁴¹⁾. In a sustainability perspective, it is important to explore available food resources at our disposal. With the expected growth in the aquaculture industry, protein-rich by-products will become even more available. Such by-products should ideally be utilized for human consumption ⁽⁴²⁾.

Bastos *et al* found that adding fish residue flour to wheat bread resulted in products with higher content of protein, essential fatty acids and minerals, and lower contents of carbohydrates ⁽⁴³⁾.

The sensory acceptancy for bread with fish residue flour were better than or as good as bread without fish flour. Groups that could benefit from enriched products are those with increased protein needs, if ensuring high-quality protein in the final product.

The strengths of the fish protein human intervention study is the randomised controlled double-blind design and the frequent follow-up of the participants. The inclusion of participants with increased risk of T2DM, and thus potentially high benefit of a supplement influencing glucose tolerance, is a strength compared to other studies on fish protein ^(25, 29, 31, 38). A strength of the study is also that we performed a postprandial uptake study to investigate that the fish protein capsules were taken up by the body. The main limitation of the present study is that we did not fulfil our power calculations indicating that 100 participants needed to complete the study to detect a clinically relevant difference between the two groups of 0.4 mmol/l in 2h-OGTT-s-glucose at the end-of-study visit. However, an increase in 2h-OGTT-s-glucose of 0.48 mmol/L (not significant) from intake of fish protein among 74 participants with increased levels of either fasting s-glucose, 2h-OGTT-s-glucose or HbA1c, decreases the risk of a type II error. In addition, the use of FFQ as a dietary registration method to register changes in the diet during the intervention must be pointed out as a limitation of the study. However, as the intervention consisted of taking a supplement and the participants were instructed not to change their dietary habits, we did not expect any dietary changes.

In conclusion, in the present study a daily intake of 7.5 g salmon fish protein did not affect glucose tolerance markers among participants with increased risk of diabetes. However, in a sustainability perspective, salmon fish protein utilized for human consumption could be a valuable protein supplement or ingredient.

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356 **Conflict of interest**

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362 **Authorship**

363 KSH, IO, KTD, TS, SMU and KBH conceived and designed the study; KSH, IO, MH, NAS, MS
364 conducted the study; KSH, IO, SMU and KBH performed the statistical analyses and interpreted
365 the results; KSH, IO, SMU and KBH wrote the manuscript and had primary responsibility for the
366 final content. All authors have critically reviewed the manuscript.

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489

490 **Figure legends**

491 **Figure 1. Flow diagram of the study participants.**

492 **Supplementary figure 1. Uptake of study product. Serum amino acids in five healthy**
493 **participants at fasting and 1h after intake of study product.**

Table 1. Characterization of the encapsulated salmon⁴⁹⁵ fish protein.

	g/100 g	mg/daily dose
Crude fat	13.2	990
Fatty acid		
SFA	2.3	173
MUFA	5.4	405
PUFA	5.1	383
n-3 fatty acids	3.2	240
EPA (20: 5n-3)	0.6	45
DHA (22: 5n-3)	1.4	105
Crude protein	69.7	5228
Amino acid profile		
Alanine	3.98	299
Arginine	4.09	307
Aspartic acid	6.04	453
Cysteine + cystine	0.78	59
Glutamic acid	7.86	590
Glycine	5.18	389
Hydroxyproline	0.89	67
Ornithine	<0.05	0
Proline	3.36	252
Serine	2.97	223
Taurine	0.72	54
Tyrosine	2.19	164
<i>Essential amino acids</i>		
Histidine	1.57	118
Isoleucine	2.59	194
Leucine	4.65	349
Lysine	4.73	355
Methionine	1.84	138
Phenylalanine	2.69	202
Threonine	2.91	218
Tryptophan	0.82	61
Valine	3.25	244

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosaheptaenoic acid

Table 2. Subject characteristics at baseline.
(Mean values and standard deviations (SD); median values and quartiles (Q1-Q3); frequencies and percentages (%)).

	Fish protein n=39	Placebo n=35
	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)
Descriptives		
Age, years	54.5 (10.2)	56.7 (11.0)
Sex, female, n(%)	24 (61.5)	23 (65.7)
BMI, kg/m ²	34.0 (5.2)	32.9 (3.9)
Daily tobacco use, n(%)	3 (7.7)	8 (22.9)
CVD history, n(%)	0 (0)	1 (2.9)
Lipid lowering drug use, n(%)	9 (23.1)	7 (20.0)
Blood pressure lowering drug use, n(%)	12 (30.8)	11 (31.4)
Blood biochemistry		
hsCRP, mg/L	3.4 (1.8-6.0)	3.3 (2.2-6.0)
Creatinine, µmol/L	65 (12)	64 (11)
eGFR, mL/min/1.73m ²	97 (13)	97 (13)
Asat, U/L	25 (7)	24 (6)
Alat, U/L	28 (21-44)	29 (23-38)
Gamma-GT, U/L	29 (20-55)	31 (20-41)
Mercury, nmol/L	6 (5-8)	7 (5-10)

CVD history includes heart attack and angina. Abbreviations: BMI, body mass index; CVD, cardiovascular disease; hsCRP, high-sensitive C-reactive protein; eGFR, estimated glomerular filtration rate; gamma-GT, gamma-glutamyl transferase

Table 3. Primary and secondary outcomes.

(Mean values and standard deviations (SD); median values and quartiles (Q1-Q3); B-coefficients and 95 % confidence intervals (95 % CI)).

		Fish protein n=39		Placebo n=35		Linear regression Change in the fish protein group relative to the placebo group			
		Baseline	Change	Baseline	Change				
	N	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)	Crude values B (95% CI)	Adjusted values B (95% CI)	[†] P	[‡] P
Fasting s-glucose, mmol/L	73	5.4 (0.5)	0.2 (0.4)	5.7 (0.6)	0.0 (0.4)	0.08 (-0.10 , 0.25)	0.02 (-0.16 , 0.20)	0.37	0.80
2h-OGTT-s-glucose, mmol/L	72	5.8 (1.4)	0.5 (1.6)	6.3 (1.5)	-0.4 (1.5)	0.61 (-0.06 , 1.27)	0.48 (-0.21 , 1.16)	0.07	0.17
Fasting s-insulin, pmol/L	73	94 (64-140)	-1 (-18-19)	110 (63-153)	-7 (-15-11)	0.06 (-0.09 , 0.21)	0.05 (-0.10 , 0.21)	0.42	0.52
2h-OGTT-s-insulin, pmol/L	73	512 (196-726)	33 (-58-250)	566 (306-710)	-29 (-218-87)	0.23 (0.01 , 0.45)	0.21 (-0.02 , 0.44)	0.04	0.07
HOMA-IR	73	3.86 (2.4-5.6)	0.02 (-0.5-1.0)	4.46 (3.0-5.9)	-0.3 (-0.7-0.6)	0.09 (-0.08 , 0.26)	0.07 (-0.11 , 0.24)	0.31	0.43
HbA1c, mmol/mol	73	41 (3.4)	0 (-1-2)	40 (3.3)	0 (-1-1)	0.01 (-0.84 , 0.87)	-0.22 (-1.09 , 0.64)	0.98	0.61

Differences between the groups in primary and secondary outcomes were tested with a linear regression model. Skewed variables (fasting s-insulin, 2h-OGTT-s-insulin and HOMA-IR) were log-transformed before analysis. The regression coefficient expresses the mean difference between the groups. A negative regression coefficient in this table represents a reduction in the fish protein group compared to the placebo group, and a positive regression coefficient represents an increase.

[†]P for difference between the fish protein group and placebo group using crude values: end-of-study values adjusted for group and baseline values.

[‡]P for difference between the fish protein group and placebo group using adjusted values: end-of-study values adjusted for group, baseline values, age, sex and weight change.

Table 4. Other pre-specified outcomes.

(Mean values and standard deviations (SD); median values and quartiles (Q1-Q3); B-coefficients and 95 % confidence intervals (95 % CI)).

		Fish protein n=39		Placebo n=35		Linear regression Change in the fish protein group relative to the placebo group			
		Baseline	Change	Baseline	Change	Crude values B (95% CI)	Adjusted values B (95% CI)	[†] P	[‡] P
	N	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)	Mean/median (SD/Q1-Q3)				
Total-C, mmol/L	74	5.4 (1.2)	-0.3 (0.6)	5.2 (0.9)	-0.1 (0.4)	-0.15 (-0.36 , 0.06)	-0.15 (-0.37 , 0.07)	0.16	0.17
LDL-C, mmol/L	74	3.8 (1.1)	-0.3 (0.4)	3.5 (0.9)	-0.1 (0.4)	-0.15 (-0.33 , 0.03)	-0.13 (-0.30 , 0.04)	0.10	0.14
HDL-C, mmol/L	74	1.3 (0.3)	0 (0.2)	1.4 (0.4)	0.0 (0.2)	-0.04 (-0.12 , 0.04)	-0.03 (-0.10 , 0.05)	0.33	0.49
Triglycerides, mmol/L	73	1.65 (1.21-2.13)	0.00 (-0.26-0.26)	1.45 (1.08-2.05)	-0.04 (-0.27-0.11)	0.07 (-0.06 , 0.20)	0.06 (-0.07 , 0.19)	0.26	0.39
Weight, kg	74	99.2 (80.7-114.9)	1.0 (-0.2-2.0)	96.0 (86.7-106.7)	0.4 (-0.8-1.3)	0.01 (-0.00 , 0.02)	0.01 (-0.00 , 0.02)	0.07	0.08 [§]
SBP, mmHg*	71	119 (13)	1.6 (8.2)	122 (16)	2.5 (11.3)	-1.7 (-6.1 , 2.8)	-1.8 (-6.4 , 2.9)	0.45	0.45
DBP, mmHg*	71	71 (10)	-0.5 (4.7)	71 (10)	-0.9 (5.7)	0.3 (-2.2 , 2.7)	-0.3 (-2.7 , 2.1)	0.82	0.79

Differences between the groups in other pre-specified outcomes were tested with a linear regression model. Skewed variables (triglycerides and weight) were log-transformed before analysis. The regression coefficient expresses the mean difference between the groups. A negative regression coefficient in this table represents a reduction in the fish protein group compared to the placebo group, and a positive regression coefficient represents an increase.

*Three participants had not taken their antihypertensive medication before one of the study visits and were excluded from the statistical analysis of SBP and DBP.

[†]P for difference between the fish protein group and placebo group using crude values: end-of-study values adjusted for group and baseline values.

[‡]P for difference between the fish protein group and placebo group using adjusted values: end-of-study values adjusted for group, baseline values, age, sex and weight change.

[§]P for difference between the fish protein group and placebo group using adjusted values: end-of-study values adjusted for group, baseline values, age and sex.

Table 5. Energy and nutrient intake.
(Median values and quartiles (Q1-Q3)).

	N	Fish Protein n=39		Placebo n=35		P
		Baseline	Change	Baseline	Change	
		Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	
Energy, kJ/d	74	9295 (7940-11760)	-559 (-1278-462)	9257 (7931-10618)	-971 (-2828-417)	0.24
Protein, E%	74	16.6 (15.9-18.7)	-0.4 (-2.5-1.3)	16.9 (15.3-18.6)	0.2 (-1.1-1.4)	0.22
Fat, E%	74	36.2 (31.6-39.9)	-0.5 (-4.3-1.9)	35.2 (33.5-38.6)	0.2 (-3.9-2.8)	0.40
Saturated, E%	74	13.5 (11.8-15.5)	0.3 (-0.7-1.4)	11.9 (11.1-15.0)	0.2 (-0.9-1.3)	0.95
Monounsaturated, E%	74	13.0 (11.7-15.2)	-0.4 (-2.3-0.7)	13.5 (11.9-14.6)	0 (-2.0-1.5)	0.32
Polyunsaturated, E%	74	5.9 (4.8-6.9)	-0.6 (-1.4-0.3)	5.8 (4.6-7.2)	0 (-1.0-0.9)	0.18
Carbohydrates, E%	74	40.2 (36.7-47.8)	1.0 (-0.8-5.2)	41.6 (35.9-45.4)	0.2 (-2.9-2.4)	0.11
Fibre, E%	74	2.4 (1.8-2.7)	0.1 (-0.4-0.2)	2.5 (2.0-3.0)	0 (-0.3-0.2)	0.56
Sugar, E%	74	5.0 (3.3-8.3)	-0.4 (-1.8-1.7)	4.4 (2.9-7.0)	0.2 (-1.2-1.7)	0.64
Alcohol, E%	74	1.7 (0.5-3.9)	0 (-0.7-0.2)	2.1 (0.5-7.5)	0 (-0.7-0.7)	0.60

Differences in energy and nutrient intake between the groups were tested with Mann-Whitney. kJ, kilojoule; E%, percentage of total energy intake.

503 **Supplementary tables**

Supplementary table 1. Uptake of study product in the postprandial study (n=5).
(Mean values and standard deviations (SD)).

	Fasting		Change		<i>P</i>
	Mean	SD	Mean	SD	
AMBA, μM	21.9	7.3	3.7	0.9	0.06
Alanine, μM	344.0	55.6	57.8	18.3	0.06
Asparagine, μM	64.5	8.9	16.6	10.4	0.13
Aspartic acid, μM	10.3	2.2	-1.3	2.0	0.31
Arginine, μM	96.8	17.5	26.6	4.8	0.06
Citrulline, μM	37.0	8.4	0.1	1.1	0.81
Creatinine, μM	88.8	15.4	-1.1	5.1	0.63
Cystathionine, μM	0.2	0.1	0.0	0.0	0.06
Cysteine, μM	296.7	24.5	-12.8	9.0	0.13
Glutamic acid, μM	30.0	9.4	-2.1	6.5	0.63
Glutamine, μM	664.0	58.4	75.4	46.5	0.06
Glutathione, μM	7.3	2.7	0.6	0.4	0.06
Glycine, μM	223.2	41.6	103.0	16.1	0.06
Homocysteine, μM	12.3	2.1	-0.8	0.5	0.06
Ornithine, μM	75.9	6.9	16.8	10.5	0.06
Proline, μM	180.5	47.5	46.2	6.1	0.06
Serine, μM	112.8	15.3	28.8	6.6	0.06
Taurine, μM	96.8	19.8	3.4	26.8	1.00
Tyrosine, μM	49.3	5.2	5.0	4.4	0.13
<i>Essential amino acids</i>					
Histidine, μM	87.1	5.4	7.8	5.3	0.06
Isoleucine, μM	77.1	5.8	17.3	6.9	0.06
Leucine, μM	146.7	13.8	25.9	12.7	0.06
Lysine, μM	197.5	39.6	49.7	15.7	0.06
Methionine, μM	26.7	4.2	6.3	2.4	0.06
Phenylalanine, μM	64.7	3.1	7.1	4.3	0.06
Threonine, μM	132.0	19.4	30.5	11.9	0.06
Tryptophan, μM	61.9	8.7	2.1	6.4	0.81
Valine, μM	257.5	17.4	18.4	26.3	0.31

Changes in serum amino acids 1h after intake were tested with Wilcoxon signed rank test. AMBA; 2-aminobutyric acid.