Health effects of supplementation with cod protein hydrolysate

Impact on glucose metabolism and appetite in healthy subjects and gut health in irritable bowel syndrome

Hanna Fjeldheim Dale

Thesis for the degree of Philosophiae Doctor (PhD)
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- Centre for Nutrition, Department of Clinical Medicine, University of Bergen, Bergen
- Section of Gastroenterology, Department of Medicine, Haukeland University Hospital, Bergen
- The Clinical Research Unit, Ålesund Hospital, Helse Møre og Romsdal, Ålesund

The main supervisor was Professor, MD Gülen Arslan Lied. Co-supervisors were Professor, MD Jan Gunnar Hatlebakk, Professor, MD Trygve Hausken and Assoc. Professor, MD Dag Arne Lihaug Hoff.

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List of abbreviations

ACE	Angiotensin-1 converting enzyme
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AUC	Area under the curve
BCAA	Branched chained amino acid
BMI	Body mass index
CCK	Cholecystokinin
CPH	Cod protein hydrolysate
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DPP-4	Dipeptidylpeptidase-4
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
FGID	Functional gastrointestinal disorder
FODMAPs	Fermentable oligo-, di-, monosaccharides and polyols
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide 1
GLUT4	Glucose transporter type 4
GOAT	Ghrelin O-acyltransferase
HDL	High-density lipoprotein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
iFABP	Intestinal fatty acid binding protein
IL	Interleukin
INF-γ	Interferon-gamma
IRS	Insulin-receptor substrate

ISL5	Insulin like peptide 5
LDL	Low-density lipoprotein
LPB	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
mTOR	Mammalian target of rapamycin
NSAID	Non-steroid anti-inflammatory drug
OGTT	Oral glucose tolerance test
PCB	Polychlorinated biphenyl
PI3K	Phosphatidylinositol 3-kinase
PUFA	Polyunsaturated fatty acid
PYY	Peptide tyrosine tyrosine
RCT	Randomized controlled trial
SCFA	Short-chain fatty acid
tAUC	Total area under the curve
T2D	Type 2 diabetes
TNF-α	Tumour necrosis factor alpha

Abstract

Background: Emerging evidence suggest that peptides from fish have several beneficial health effects in the human body. A huge part of the world's population are affected by life-style diseases related to overweight and obesity, leading to impaired glucose metabolism and other adverse metabolic impairments. In addition, around one out of ten individuals are affected by irritable bowel syndrome (IBS), a diagnosis that most often is only partly controlled by dietary changes and/or pharmacological treatment and cause reduced quality of life. A dietary supplement contributing to increased utilization of residual material from the fishing industry, capable of improve health outcomes related to metabolism and gastrointestinal function, can arguably be regarded valuable both for those individuals affected as well as contribute to a more sustainable industry.

Aim: To investigate the effects of a supplement with cod protein hydrolysate (CPH) on glucose metabolism and appetite in healthy subjects, and on gut health in patients with IBS.

Method: Three randomized double-blinded clinical intervention studies were conducted. Study 1 was a crossover trial in 41 healthy, middle-aged to elderly adults, and included two study days, with 4-7 days wash out in between. The intervention consisted of 20 mg of CPH (or control) per kg body weight, given before a standardized breakfast meal. Study 2 was a dose-range study in 31 healthy, older subjects receiving four different doses of CPH equivalent to 10, 20, 30 or 40 mg/kg body weight in random order, each dose taken daily for one week, with a week of wash-out in between. Primary outcome in both studies was postprandial response in glucose metabolism, measured by samples of serum glucose, insulin and plasma glucagon-like peptide 1 (GLP-1) (Paper I and III), and secondary outcomes (Study 1) were postprandial acylated ghrelin concentration and sensations associated with appetite (Paper II). Study 3 included 28 patients with IBS randomly allocated to daily supplementation with 2.5 g CPH (n=13) or placebo (n=15) for 6 weeks. Outcomes were evaluated at baseline and after six weeks.

Primary outcomes were symptom severity evaluated by IBS Severity Scoring System (IBS-SSS) and quality of life. Secondary outcomes included gut integrity markers (zonulin, lipopolysaccharide-binding protein, intestinal fatty acid binding protein) and pro-inflammatory cytokines in serum, fecal calprotectin and fecal fermentation measured by concentration of short-chain fatty acids (SCFAs) (Paper IV).

Results: No differences were observed between CPH and control for postprandial concentrations of glucose, GLP-1, acylated ghrelin or sensation related to appetite, but the postprandial insulin concentration was significantly lower after CPH compared to control (Study 1). No differences in estimated postprandial maximum level of glucose, insulin or GLP-1 were observed when comparing the dose of 10 mg/kg body weight of CPH to 20, 30 or 40 mg/kg body weight, but the estimated maximum value of glucose and insulin was lower for the 40 mg/kg body weight dose than the 10 mg/kg body weight dose (Study 2). Total IBS-SSS scores were reduced in both the CPH group and the placebo group, with no significant differences between intervention and placebo treatment. Concentrations of serum markers and SCFAs did not change for any of the groups (Study 3).

Conclusion: Study 1 demonstrated that a single dose of CPH before a breakfast meal reduced the postprandial insulin concentration, without affecting blood glucose response, GLP-1 levels, concentrations of acylated ghrelin or sensations related to appetite in healthy individuals. Study 2 demonstrated that serum glucose and insulin concentrations tended to be reduced with increasing doses of CPH, however no significant effects were observed. Study 3 suggested that 2.5 g of CPH taken daily by IBS patients for six weeks did not affect symptom severity, gut integrity markers, inflammatory markers or fecal fermentation when compared to placebo.

List of publications

I. Dale HF, Jensen C, Hausken T, Lied E, Hatlebakk JG, Brønstad I, Hoff DAL, Lied GA.
Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: a double-blind cross-over trial
J Nutr Sci. 2018;7:e33. Epub: 28/11/2018.

II. Dale HF, Jensen C, Hausken T, Lied E, Hatlebakk JG, Brønstad I, Hoff DAL, Lied GA.
Acute effect of a cod protein hydrolysate on postprandial acylated ghrelin concentration and sensations associated with appetite in healthy subjects: a double-blind crossover trial
Food Nutr Res. 2019;63. Epub: 07/11/2019.

III. Jensen C, Dale HF, Hausken T, Lied E, Hatlebakk JG, Brønstad I, Lied GA, Hoff DAL.
Supplementation with cod protein hydrolysate in older adults: a dose range cross-over study
J Nutr Sci. 2019;8:e40. Epub:02/12/2019.

IV. Dale HF, Jensen C, Hausken T, Hatlebakk JG, Brønstad I, Valeur J, Hoff DAL, Lied GA.
Effects of a cod protein hydrolysate supplement on symptoms, gut integrity markers and fecal fermentation in patients with irritable bowel syndrome
Nutrients. 2019;11(7):e1635. Epub: 20/07/2019.

Disclaimer

Two of the clinical studies included in the current PhD-thesis are carried out in cooperation with Caroline Jensen, a colleague and fellow PhD-candidate at Centre for Nutrition, University of Bergen. Paper I and III in this thesis holds shared first-authorship with Caroline Jensen. These papers report major findings important for the understanding of the overall project, hence they will also be included in the PhD-thesis of Caroline Jensen, planned to be submitted spring 2020.

Contents

1. Introduction

1.1 Fish – A great source of high-quality protein

1.1.1 Fish consumption and health effects

The Norwegian Health Authorities recommend a fish intake of 300-450 g a week, corresponding to two to three dinner meals.[1] The Norwegian population eat on average less fish and more red meat than recommended.[2] According to all the well-known benefits of reducing meat intake, an increase in fish intake will in most western populations arguably be beneficial both in regards to human health, environmental footprint and sustainable food production.[3,4]

Fish contains 20-30% protein, a varying amount of fat and is a good source of micronutrients such as vitamin D, vitamin B12, iodine and selenium. Besides being a good source of high-quality protein, fish is considered the main source of the essential long chain omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[5] It is classified according to the content of fat, and fish species containing less than 2% fat is considered lean fish, species containing 2-5% fat is considered to be medium fatty fish, whereas species with a fat content above 5% is classified as fatty fish.[5]

Currently, the Norwegian Health Authorities recommend a weekly intake of 200 g fatty fish.[1] However, a comprehensive risk-assessment from the European Food Safety Authorities (EFSA) published in November 2018, evaluating the health risk of exposure to dioxins and dioxin-like polychlorinated biphenyls (PCBs) from foods, led to a drastically reduction in the tolerable weekly intake (TWI) for dioxins and PCBs.[6] As fatty fish is one of the main dietary sources to these detrimental substances, a new risk-benefit evaluation regarding consumption of fatty fish is needed to evaluate whether the recommendations from the Norwegian Health Authorities needs adjustments.[7]

The great interest in the health effects of fish consumption have escalated from the 1970s, when it was observed that the Greenland Eskimos, a population with a high intake of fish, had a low prevalence of cardiovascular disease (CVD) and type 2 diabetes (T2D).[8] This observation is considered the first indication of the positive health effects of marine omega-3 PUFAs and has generated comprehensive investigations of the relationship between fish consumption and the beneficial effect of omega-3 PUFAs. Later observational studies have found fish consumption to be associated with reduced risk of coronary heart disease and stroke,[9-12] but the effect on diabetes is more inconsistent.[13-18] Consumption of fatty fish with a high concentration of omega-3 PUFAs is associated with a reduction of circulating triglycerides, but the effect on cholesterol is more inconclusive.[19] Regular fish consumption is associated with decreased levels of inflammatory markers related to CVD, hence is thought to have an anti-inflammatory effect.[20] Although the mechanisms behind the anti-inflammatory effect is not fully understood, it has been suggested that it is a result of a reduced production of pro-inflammatory eicosanoids derived from arachidonic acids and increased conversion of EPA and DHA to eicosanoids with anti-inflammatory properties.[21]

1.1.2 Health effects of lean fish intake

The beneficial health effects of regular fish consumption have traditionally been attributed the content of marine long-chain omega-3 PUFAs and their importance for cardiovascular health. Interestingly, emerging evidence from studies reporting on the effects of lean fish and fish proteins in particular, eliminates the unique effect of the omega-3 PUFAs and suggests that other fish components besides the healthy fatty acids may improve health.[22] Several observational studies in humans have indicated that intake of lean fish may beneficially influence metabolic and cardiovascular health. Consumption of lean fish has been associated with beneficial changes in body composition, glucose regulation and lipid metabolism[23,24], in addition to reduced risk of metabolic syndrome and T2D.[14-17,25] These associations are supported by several animal

studies reporting on beneficial effects of cod protein on glucose regulation and insulin sensitivity,[26-28] lipid metabolism[29] and hypertention.[30]

In addition, promising results are reported in several clinical trials in humans, investigating health effects of an intervention with lean fish, however the overall results are conflicting. Most studies have compared lean fish with fatty fish or a non-seafood diet, containing equal amounts of proteins from lean meat, eggs, chicken and dairy products. A beneficial effect of lean fish consumption on lipid status has been reported in some interventions in healthy individuals,[31,32] whereas no effect or a negative effect on lipid concentrations has been reported in others.[33-36] Two studies have reported a beneficial effect of lean fish on glucose metabolism in normal-weight and overweight individuals,[37,38] whereas one study in overweight and obese individuals reported a beneficial effect of salmon, but not cod.[39] Lean fish has been reported to reduce blood pressure in CVD patients,[40] but the same effect was not seen in a group of overweight and obese individuals.[41]

Overall, existing literature point towards several beneficial health effects of consuming lean fish, however the limited number of clinical trials emphasize the need for more research to better understand the potential mechanisms generating these beneficial health outcomes.

1.1.3 Fish and protein quality

It is well established that an adequate amount of proteins in the diet is important to maintain a healthy body. The recommended daily protein intake for healthy adults is between 0.8-1.5 g per kg body weight, dependent on age and level of physical activity.[42] The nutritional value of proteins in different foods is dependent on several factors, including primary structure, susceptibility to enzymatic digestion, chemical changes during processing, the composition of amino acids and the overall content of essential amino acids. Essential amino acids are amino acids the human body are not capable of producing, thus they must be added in the diet. Of the 20 existing amino acids, eight are

essential. Foods regarded as a source to high-quality proteins holds a high content of the essential amino acids.[43]

Fish and marine resources, such as by-products from the fishing industry, are great sources of high-quality protein.[44] Fish proteins contains all the essential amino acids, with a particularly high content of lysine and leucine. Of the non-essential amino acids, aspartic, glutamic acid and alanine are present in quite high amounts in proteins from fish. In addition, fish holds a high content of the amino acid-derived organic acid taurine.[45] Most seafood proteins have a high digestibility, normally above 90%, thus the exploitation of the available amino acids in the protein source is good.[43]

1.1.4 Utilization of marine resources: Fish protein hydrolysates

Given the increased global demand for sources of high-quality protein, as well as the requirement for sustainable processing and production in the industry, it is an increasing interest in extraction of nutrients from by-products from the fishing industry. Better utilization of protein rich residual material from the industry can arguably be regarded both environmentally friendly, sustainable and cost-effective.[44] Additionally, in coast located countries with an established fishing industry, such as Norway, a special interest is given the potentially biologically active effect of peptides generated from hydrolyzing fish by-products.[44]

Various biotechnological approaches are currently investigated aiming to extract valuable nutrients and bioactive compounds from fish and fish by-products, capable of enhancing human health. Proteins from fish can be broken down to fish protein hydrolysates by enzymatic conversion of intact protein into peptides. Usually, these protein fragments, referred to as peptides, contains less than 20 amino acids.[44] Intervention studies evaluating the possible health effects of lean fish, have this far mostly focused on the possible health effects of cod filet, which in dry weight accounts for 50-70% of the whole fish. The remaining part of the fish is not suitable for human consumption as is, and is referred to as residual- or waste-material. Recently, improved processing technologies enable better utilization of this protein-rich residual material,

which after freeze drying contains approximately 70% protein.[46] Peptides isolated from fish proteins by hydrolysis can be derived from both muscle, skin, scale, bones and other tissue, and can with right processing become high-quality protein powders suitable for human consumption.[47] Existing literature have reported using several different enzymes utilized for food-processing in the industry, such as trypsin and Corolase®, among others, in the production of fish protein hydrolysates.[48,49] These fish protein hydrolysates are of commercial interest, due to their good nutritional composition, beneficial amino acid profiles and possible bioactive properties.[50] Accordingly, if peptides derived from hydrolysis of residual material from the fishery industry is found to be suitable for human consumption and capable of improving different health outcomes, it will innovate both human health and the industry.

1.1.5 Fish proteins as a source of bioactive peptides

In addition to being a good source of amino acids and energy, some dietary proteins are suggested to have a number of other important effects beyond nutrient supply, most often linked to the effects of possible bioactive peptides.[51] Bioactive peptides can be formed naturally from dietary proteins by digestion and enzymatic degeneration in the gut or by microbial fermentation, or they can be delivered in the diet as already hydrolyzed proteins. Bioactive peptides tend to have 2 to 20 amino acid residues, present as di- and tripeptides with low molecular mass.[52] Bioactivity is linked to the presence of different combinations of amino acid sequenced, with a possibly unique potential to beneficially modulate different metabolic pathways and thereby contribute to disease modulation.

Different metabolic properties have been linked to different amino acid sequences present in fish peptides.[44] Some are suggested to reduce hypertension by inhibit angiotensin-1 converting enzyme (ACE), some to beneficially alter the blood glucose metabolism through different mechanisms, such as inhibiting dipeptidylpeptidase-4 (DPP-4), and some are suggested to affect the gut microbiota by contributing to increased conjugation of bile acids.[53] The peptides are hypothesized to be effective by

generating a local effect directly in the gastrointestinal (GI) tract, or after entering the circulation by absorption in the gut.[54] Some of the suggested effects of bioactive peptides from fish are depicted in **Figure 1.**

The use of natural bioactive products has for a long time been used for prevention and treatment of a wide spectrum of conditions, but based on current evidences the scientific validity of such products is limited. However, it is sufficient preliminary data to indicate that bioactive compounds may potentially be valuable for clinical use, thus further clinical trials investigating these effects are of great need.[55]

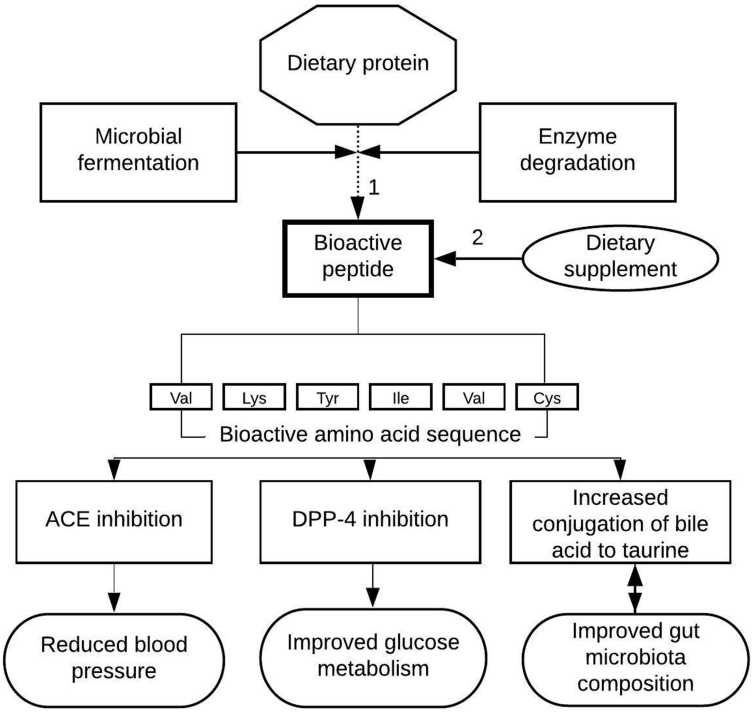


Figure 1. Suggested metabolic effects of bioactive peptides from fish. Peptides holding amino acid sequences with potential bioactive effects are suggested to influence different pathways involved metabolic regulation. Bioactive peptides can be formed after ingestion of food proteins by degeneration by enzymes in the gut or microbial fermentation (1) or they can be added in the diet as a nutrition supplement containing hydrolyzed protein (2). Reprinted from Dale et al 2019[22] with approval from publisher. ACE: Angiotensin-1 converting enzyme, DPP-4: dipeptidylpeptidase-4.

1.1.6 Results from animal studies with fish protein hydrolysates

Several animal studies have investigated the effect of fish protein hydrolysates on different metabolic outcomes, using hydrolysates based on different fish species. Most studies have compared the effect of fish protein hydrolysates with whole casein.

A protein hydrolysate from salmon have been reported to improve the lipid profile in rats by reduce total plasma cholesterol, increase the high-density lipoprotein (HDL):total cholesterol ratio and lowering acyl-CoA-cholesterol acyltransferase activity, when compared to protein from casein.[56] Additionally, a study including rats fed a high-fat diet with either casein or salmon protein hydrolysate as source of protein, reported that the rats fed fish protein hydrolysate became resistant to high fat diet-induced obesity.[57] In addition, they found the rats fed fish protein hydrolysate to have reduced postprandial plasma glucose and triglycerides levels, as well as lower triglycerides levels accumulated in the liver compared with the casein fed rats.[57] A similar effect has been reported from a study in rats investigating a fish protein hydrolysate from saithe, where the fish protein hydrolysate (reported to have a high content of taurine and glycine) was found to reduce visceral adipose tissue mass and elevate fasting bile acids compared to casein.[58] These findings are supported by a later study, reporting that a protein hydrolysate from salmon beneficially altered the fatty acid composition in liver and adipose tissue in a mouse model of chronic inflammation.[52]

In contrast, more conflicting results are presented in a study investigating the effect of two different fish protein hydrolysates, based on either herring or salmon.[59] In this study, the herring-fed rats presented lower serum HDL- and low-density lipoprotein (LDL)-cholesterol, as well as higher triglycerides than the casein/whey-fed rats, implying a negative effect of herring on lipid status. The salmon-fed rats gained more weight and had improved postprandial blood glucose regulation than the casein/whey-fed rats.[59] The same fish protein hydrolysates were later reported to contain several peptide sequences with possible ACE-inhibiting activities,[60] supported by another study reporting that a diet with cod residual proteins prevented hypertension in rats.[30] The authors stated that the mechanistic effects behind this observation are unknown, but

suggested that the cod residual proteins might contain amino acid sequences with ACE and/or renin inhibitory effects, capable of effecting the blood pressure regulating renin-angiotensin system.[30]

Overall, the findings from animal studies investigating the metabolic effects of different fish protein hydrolysates report several beneficial effects of these peptides, however the results are somehow conflicting, and it is a lack of knowledge regarding the mechanistic effects causing these potential beneficially alterations.

1.1.7 Results from human interventions with fish protein supplements

Several clinical interventions have investigated the potential effects of supplements with low doses of fish proteins or fish protein hydrolysates in humans on different outcome-measures related to metabolic and gastrointestinal health. The relevant studies are presented in **Table 1.**

Three studies have evaluated the effect of a supplement with fish proteins or fish protein hydrolysates on glucose and lipid metabolism in overweight and obese individuals.[47,61,62] Vikøren et al. reported 8 weeks of supplementation with 3 or 6 g cod protein hydrolysate in 34 overweight/obese adults to result in lower values for fasting glucose, lower postprandial glucose concentration, lower LDL cholesterol, reduced body fat and increased muscle mass compared to baseline.[61] Vildmyren et al. reported 8 weeks of daily supplementation with 6 g protein from cod residual material to beneficially alter the postprandial serum concentrations of non-esterified fatty acids in 42 healthy overweight or obese adults, when compared to placebo.[47] Hovland et al. compared the effects of 8-weeks supplementation with 2.5 g of either cod protein, herring protein hydrolysate, salmon protein hydrolysate or milk proteins (90% casein and 10% whey) in 77 overweight/obese adults.[62] They reported the cod protein supplement to reduce postprandial glucose concentration within the group, but no significant differences were observed when compared to the salmon- or herring hydrolysate groups.

Table 1. Overview of studies investigating the effect of supplementation with low doses of fish proteins and fish protein hydrolysates on different health outcomes related to metabolism and risk markers for life-style diseases, body weight and gut health.

Primary outcomes	Author Year	Subjects	Design	Duration	Test material	Placebo	Results
Glucose and lipid metabolism	Vikøren et al.[61] 2013	34 overweight / obese adults	RCT Parallel-group	8 w	3 and 6 g cod PH	Malto-dextrin, fructose	↓ Postprandial glucose ↓ Fasting glucose ↓ LDL ↓ Body fat ↑ Muscle mass
	Vildmyren et al.[47] 2018	42 overweight / obese adults	RCT Parallel-group	8 w	6 g cod presscake meal (PC) or cod-PC-stickwater	Cellulose	Cod-PC: ↓ Postprandial serum NEFA
	Hovland et al.[62] 2019	77 overweigh / obese adults	RCT Parallel-group	8 w	2.5 g cod protein, herring PH, salmon PH	Whey and casein	All fish PHs: ↓ Postprandial glucose No effect on insulin or lipid concentrations
Body weight	Nobile et al.[63] 2016	120 overweight individuals	RCT Parallel-group	13 w	1.4 g or 2.4 g saithe PH	Whey protein	Both doses: ↓ Body weight ↓ Fat mass ↑ Plasma CCK ↑ Plasma GLP-1
Appetite	Zaïr et al.[64] 2013	15 overweight woman	RCT Cross-over	2 w	2 g blue whiting PH	2 g cellulose	↓ Postprandial glucose ↓ Sweet cravings
Hypertension	Kawasaki et al.[65] 2000	29 mild hypertensive subjects	RCT Parallel-group	4 w	100 ml drink with 3 mg sardine PH	100 ml placebo drink	↓ Blood pressure ↑ Angiotensin 1
Inflammation	Drotningsvik et al.[66] 2019	24 nursing home residents	RCT Parallel-group	6 w	5.2 g blue whiting PH in soft drink	Soft drink	↓ MCP-1 ↑ CRP
Gut health	Marchbank et al.[67] (2008)	8 healthy subjects	RCT Cross-over	1 w	50 mg NSAID + 1 g pacific whiting PH	50 mg NSAID + 1 g rice flour with 1% sea cucumber	↓ NSAID-induced permeability after fish PH
	Wu et al.[68] 2016	60 gastric cancer patients on chemo-therapy	RCT, Parallel-group	4 w	2 g cod skin peptide	2 g starch	↑ QoL ↓ Chemotherapy-induced hematologic toxicity

RCT: Randomized controlled trial, w: weeks, d: days, PH: Protein hydrolysate, LDL: Low-density lipoprotein, NEFA: Non-esterified fatty acids, CCK: Cholecystokinine, GLP-1: Glucagon-like peptide-1, MCP-1: Monocyte chemoattractant protein-1, CRP: C-reactive protein, NSAID: Non-steroid anti-inflammatory drug, QoL: Quality of life

No differences were observed from baseline to after intervention for either insulin or lipid concentrations. The authors concluded that 2.5 g of fish proteins a day may be sufficient to improve glucose regulation.[62] Taken together, the results from these three studies indicate that supplementation with relatively low doses of fish proteins- and hydrolysates are capable of beneficially alter glucose and lipid metabolism in overweight and obese subjects.

Two studies have reported on the effects of fish protein hydrolysate supplements on body weight and appetite.[63,64] Nobile et al. investigated the effect of a 13 weeks intervention with either 1.4 g or 2.8 g fish protein hydrolysate from saithe in 120 overweight individuals, and reported both doses to reduce body weight, body mass index (BMI) and fat mass, as well as waist-, thighs- and hip circumference when compared to placebo.[63] The fact that the effect was equal for both of the intervention dosages, points towards a beneficial metabolic effect of hydrolysates from fish, also when administered in low does. This is supported by a crossover study by Zaïr et al. investigating the effect of a 2-week intervention with 2 g fish protein hydrolysate from blue whiting a day in 15 overweight women, reporting that the fish hydrolysate significantly suppressed appetite when compared to placebo. Postprandial measures of glucose concentrations and self-reported sensations related to appetite showed that sweet-carvings, as well as plasma glucose levels, were reduced after the fish protein hydrolysate intervention.[64]

One study have reported beneficial effects of a fish protein hydrolysate on blood pressure.[65] Kawasaki et al. investigated a sardine muscle hydrolysate containing a suggested bioactive valyl-tyrosine motif. The hydrolysate was supplemented to 29 mild hypertensive subjects daily for 4 weeks and was reported to reduce blood pressure and increase plasma angiotensin I when compared to placebo.[65]

A feasibility pilot study performed in twenty-four nursing home residents investigated the effects of six weeks supplementation with 5.2 g protein from blue whiting compared to placebo on parameters related to inflammation and glucose metabolism.[66] They reported decreased serum concentrations of monocyte chemoattractant protein-1 (MCP-

1) and increased C-reactive protein (CRP) in the intervention group compared to placebo, and no observed effect on glucose metabolism.[66]

A few studies have reported on the effects of fish protein hydrolysates on gut health. It has been suggested after *in vitro* observations that hydrolysates from fish may have an immune-modulating effect with several beneficial properties in the intestine.[69] This hypothesis is supported by a small clinical trial reporting that a fish protein hydrolysate may prevent injuries caused by the use of non-steroid anti-inflammatory drugs (NSAIDs), change permeability and possibly prevent injurious conditions in the gut.[67] Also, a cod skin peptide has been shown to reduce chemotherapy-induced toxicity and hence improve the quality of life in gastric cancer patients.[68] These findings pose novel questions on whether a protein hydrolysate from fish can improve gut health, that needs further investigations before conclusions can be made.

The last literature search for publications related to health effects of fish protein- and fish protein hydrolysate supplements in animal and human trials relevant for this thesis was conducted in November 2019.

1.2 Body weight and metabolic implications

1.2.1 Overweight and obesity

Health challenges related to an unhealthy lifestyle have increased in a great extent the last decade. Overweight and obesity affects today in total over a third of the world´s population, with the prevalence constantly increasing.[70] Overweight and obesity is defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation that may impair health.[71] BMI is the weight-for-height index used to classify overweight and obesity in adults, measured as weight in kilograms divided by the square of height in meters. Overweight is defined as BMI ≥ 25 kg/m^2 and obesity as BMI ≥ 30 kg/m^2.[72] Individuals with increased body weight are at higher risk for the development of lifestyle related diseases, such as CVD and T2D.[73] Metabolic syndrome is an increasingly common clinical condition, defined as a cluster of risk factors for the development of CVD and T2D. The condition is recognized by metabolic abnormalities such as abdominal obesity and elevated waist circumference, dyslipidemia (with elevated triglycerides and/or reduced HDL cholesterol), hypertension and insulin resistance.[74]

The global increasing obesity problem calls for new nutritional strategies including both preventive and treatment options.[75] Weight loss is theoretically easy; the energy intake has to be less than the energy expenditure. However, the practical implementation is challenging, and continue to lack compliance.[71] One of the most obvious challenges in weight loss is hunger, which makes it difficult to maintain the dietary control.[76] Appetite is regulated by tuned interactions between the GI tract, the adipose tissue and hypothalamus, and is controlled by several different hormones promoting or inhibiting the feeling of satiety.[77] Ghrelin, a small appetite-stimulating peptide secreted from neuroendocrine cells in the stomach, is this far the only identified hormone known to stimulate hunger.[78] This quality has created the idea that compounds capable of inhibiting the act of ghrelin may be effective in the prevention and/or treatment of overweight and obesity.[79]

1.2.2 Glucose metabolism

In a fasting state and under normal physiological conditions, the plasma glucose levels are normally maintained within the narrow range from 4.0 to 6.0 mmol/L. The tight blood glucose control is balanced by glucose absorption from the intestine, production by the liver and uptake and metabolism of glucose by muscle and adipose tissue, regulated by the two hormones insulin and glucagon.[80]

Insulin is the primary regulatory hormone essential for regulation of blood glucose concentration. The hormone is produced by the pancreatic β-cells and increase the glucose uptake in peripheral tissue, as well as inhibit the production of glucose from the glycogen stores in the liver. In addition, insulin stimulate cell growth and differentiation, as well as promote the storage of substrates in the liver, muscle and fat tissue by stimulating lipogenesis and protein synthesis and inhibit lipolysis and protein breakdown.[80]

Increased glucose levels in the blood after ingestion of food triggers secretion of insulin. Insulin increase the glucose uptake in the cells by stimulating translocation of the glucose-transporter type 4 (GLUT4) from intracellular sites to the plasma membrane of the cell.[81] GLUT4 is stored in vesicles inside the cell that continuously cycles from the intracellular stores to the cell surface. The insulin receptor is part of a subfamily of the tyrosine kinases, that also includes the insulin receptor-related receptor (IRR) and the insulin-like growth factor (IGF)-1 receptor. These tyrosine kinase receptors are proteins with two α- and two β-units, that undergo autophosphorylation and catalyzes the phosphorylation of intracellular proteins.[80] At least nine intracellular proteins of the insulin/IGF-1 receptor kinases have been identified, of which four belongs to the insulin-receptor substrate (IRS) family. Upon phosphorylation by tyrosine, these intracellular IRS proteins interact with signal molecules involved in a series of signal pathways. The act of insulin on cells in peripheral tissue involves the activation of several different kinases, including phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) and mammalian target of rapamycin (mTOR).[80]

The muscle is responsible for approximately 90% of the insulin-stimulated glucose uptake and stores the glucose as glycogen. Although the adipose tissue only counts for 10% of the insulin-stimulated glucose uptake, this fraction is important for the control of the energy homeostasis in the whole body.[81] The adipose tissue play an important part in metabolic regulation and insulin resistance. In the adipose tissue, the energy is stored as triglycerides, and free fatty acids (FFAs) released from adipocytes reduce the uptake of glucose in muscle cells as well as the insulin secretion from β-cells in the pancreas. In addition, the FFAs induce glycogenolysis in the liver, leading to elevated blood glucose concentrations. In addition, the adipocytes secrete adipokines such as leptin and adiponectin, hormones involved in the regulation of food intake, energy expenditure and insulin sensitivity.[80]

1.2.3 Insulin resistance

Insulin resistance or deficiency, as seen in metabolic syndrome and diabetes, results in dysregulation of the blood glucose concentrations leading to elevated fasting and postprandial blood glucose concentration.[80] T2D is a chronic metabolic disorder characterized by increased plasma glucose levels, due to a defect in the ability of muscle and adipose tissue to appropriately respond to insulin. The insulin resistance of obesity and T2D is characterized by defects in many levels of the glucose metabolism and involves a decrease in the deposition of glucose to peripheral tissues, an overproduction of glucose in the liver as well as a functional damage of the pancreatic B-cells responsible for insulin production.[82] Mechanisms suggested to be involved in insulin resistance caused by excess of adipose tissue (as seen in overweight and obesity), leading to hyperglycemia, is depicted in **Figure 2.**

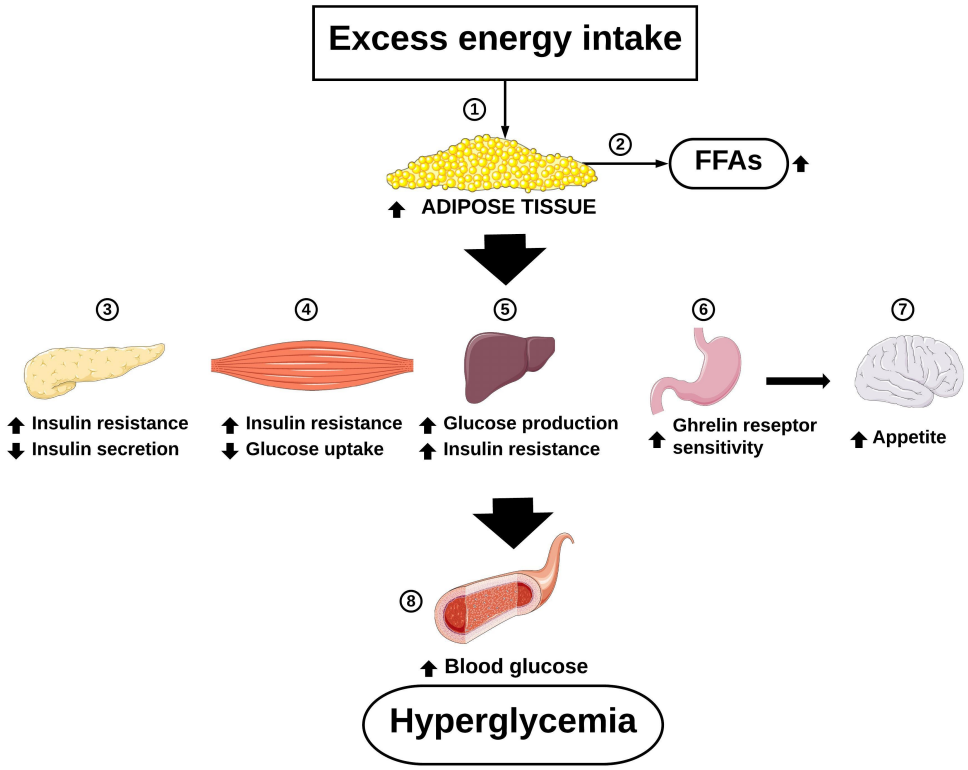


Figure 2. (1) Excess of energy intake compared to energy expenditure over time leads to an increase in adipose tissue, and potentially overweight and obesity. An increase in adipose tissue as seen in overweigh/obesity leads to (2) increased circulating concentrations of free fatty acids (FFAs), an event that is suggested to contribute to several adverse metabolic implications in pathways involved in the blood glucose metabolism, such as (3) reduced insulin production/secretion and increased insulin resistance in pancreas, (4) increased insulin resistance and decreased glucose uptake in skeletal muscles, (5) increased insulin resistance and glucose uptake in the liver and (6) increased receptor sensitivity to ghrelin action, potentially generating (7) increased appetite. Taken together, these adverse metabolic events contribute to increased concentration of glucose in the blood (8), known as hyperglycemia. The medical images are taken from Smart Servier Medical Art. Figure by Dale 2019.

Insulin sensitivity is influenced both by genetic and acquired factors. Increased adipose tissue due to overweight and obesity is the primary risk factor for insulin resistance, as the increase in adipose tissue results in elevated levels of circulating FFAs as well as inflammatory cytokines like tumor necrosis factor-alfa (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). The elevated FFAs in the circulation contribute to insulin resistance by inhibit the uptake of glucose, glyconeogenesis and oxidation of glucose as well as increase the glycogenolysis in the liver and thereby the hepatic glucose output.[80] Additionally, it occurs a reduction of the enzyme activity of important enzymes involved in the normal insulin signaling pathway, such as PI3K and protein kinase B (Akt). When insulin resistance is combined with defects in the glucose-stimulated secretion of insulin from the pancreatic β-cells it will lead to the development of impaired glucose tolerance, hyperglycemia and eventually T2D.[83]

Translocation of GLUT4 to the cell surface is crucial for the uptake of glucose, and this translocation, among other mechanisms, is impaired in T2D and contribute to the increased blood glucose concentration.[81] In the muscle cell, the translocation of GLUT4 is dependent on mTOR. It is shown that stimulation of this pathway improve glucose tolerance in animal models of T2D, but whether activation of the mTOR-pathway can improve the condition of human individuals with T2D is still not clear.[84]

1.3 Appetite

1.3.1 Neuroendocrine regulation of appetite

The energy homeostasis and regulation of appetite involves a complex network of peripheral and hypothalamic signals.[79] Hypothalamus is the main regulatory organ of the endocrine system in humans, integrating peripheral signals about dietary intake and energy expenditure from the brainstem and other centers in the brain.[85] Hypothalamus contains a number of different nuclei connected to the circulatory energy-homeostasis-regulating system, of which the arcuate nucleus (ARC) play the most important role in regulation of appetite. When food is ingested, sensory information is sent from the GI tract to the central nervous system (CNS) and the ARC, either by the Vagus nerve or by GI hormones through the bloodstream. This feedback communication between the GI tract and the regulatory appetite centers within the CNS is part of the pathway referred to as the "Gut-brain-axis".[85]

1.3.2 Gut hormones

Several gut hormones are known to be involved in the regulation of appetite. These includes ghrelin, insulin-like peptide 5 (ISL5), glucagon like-peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK) and peptide-tyrosine-tyrosine (PYY), among others.[86,87] These GI hormones are normally referred to as incretins. They are secreted precisely to regulate appetite and satiety according to composition and size of a meal. Hormones produced in the GI tract have either an appetite stimulating (orexigenic) or appetite suppressing (anorexigenic) effect on food intake.[86] An overview of some of the important hormones known to be involved in the regulation of appetite and released from the GI tract, is shown in **Figure 3.**

Ghrelin is a small appetite-stimulating peptide consisting of 28 amino acids, secreted mainly from neuroendocrine D/P1-cells in the submucosal layer of the proximal part of the stomach, and is so far the only well-known identified orexigenic hormone.[88,89]

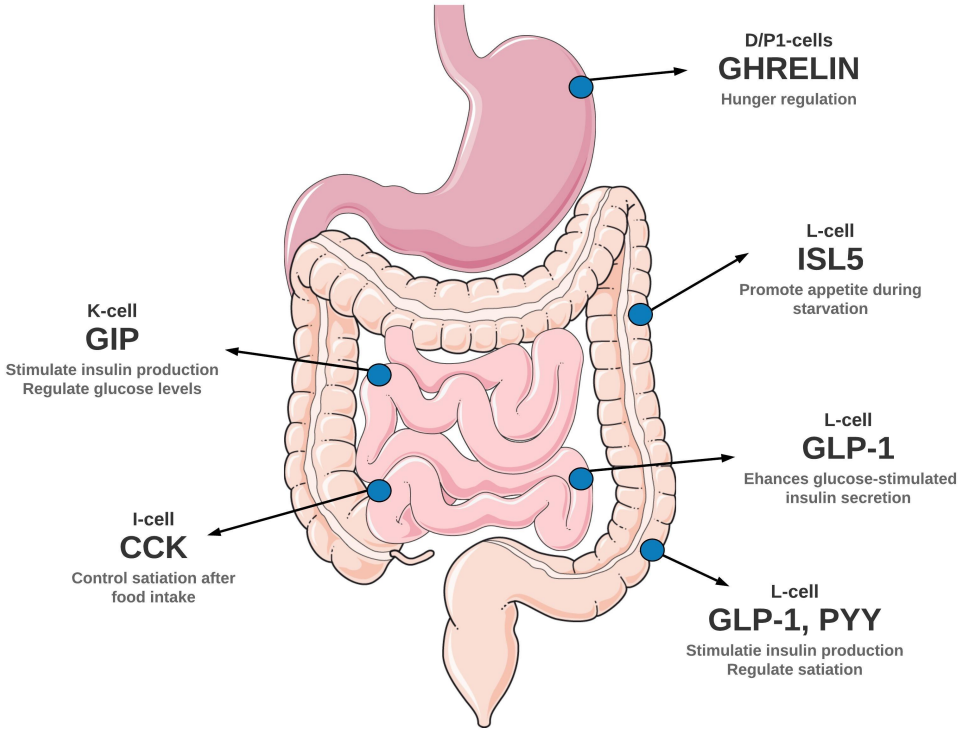


Figure 3. Overview of the production and release of some of the different appetite regulating gastrointestinal hormones produced in the stomach and the intestines. The medical images are taken from Smart Servier Medical Art. Figure by Dale 2019.

The circulating concentration of ghrelin decreases with food intake and increase with hunger.[90] The hormone exists in the circulation both as an acylated and non-acylated form. The enzyme responsible for the acylation of ghrelin is ghrelin O-acyltransferase (GOAT).[91] The acylated form of ghrelin act as a natural ligand of the growth hormone secretagogue receptor, also called the ghrelin receptor, and is therefore capable of stimulate the secretion of growth hormones, reduce the insulin secretion and impair the glucose metabolism.[78] Due to its properties, ghrelin has become a target in appetite control and treatment of obesity, but a substance inhibiting the action of ghrelin, and thereby suppressing appetite, is still not discovered.

Insulin-like peptide 5 (ISL5), a hormone produced by enteroendocrine L-cells in colon and rectum, has been identified as the second orexigenic hormone, thought to promote appetite during conditions of energy deprivation.[87] However, nor the exact physiological mechanism or the orexigenic properties of this novel hormone has yet been defined.

The two incretins GLP-1 and GIP, are peptide hormones secreted from the gut right after the ingestion of food, contributing to rapid distribution of nutrients postprandially by stimulating the release of insulin.[92] These incretins are released from the gut by a glucose-stimulated response and are thought to account for 50-70% of total insulin secretion postprandially. GLP-1 is released from the L-cells in the small intestines as well as colon, whereas GIP is primarily released from K-cells in the small intestine. GLP-1 promotes lipolysis, slows the gastric emptying, inhibit glucagon secretion in a glucose-dependent manner, and is shown to improve postprandial glycemic control in patients with T2D, as well as promote satiety.[85,92] GIP promotes energy storage in adipose tissue and enhance the formation of bone by stimulating osteoblast proliferation.[92] GIP and GLP-1 are rapid degenerated by the enzyme dipeptidyl peptidase-4 (DPP-4), a discovery leading to the development of degradation-resistant GLP-1 receptor agonists and DPP-4 inhibitors for the treatment of T2D.[92]

CCK is released from type I enteroendocrine cells in the mucosa of duodenum and jejunum immediately after the intake of nutrients, mostly after the intake of meals high in lipids and proteins. CCK has an anorexigenic effect, and once released in response to food intake it acts by activating the CCK receptor located in the vagal nerves in the GI tract.[93] In addition to inhibit food intake by induce increased feeling of satiety, CCK interacts with the other incretins in order to regulate the energy balance.[94]

PYY is another anorexic hormone released from enteroendocrine L-cells found predominantly in the distal GI tract.[95] PYY is released from the gut in a caloric-dependent manner in connection with a meal, and is shown to reduce the appetite and hence the intake of food.[94] As with CCK, meals high in fat and protein are shown to particularly stimulate the release of PYY.[96]

Recently, it has been shown that bariatric surgery leads to significant modifications in the composition of gut hormones, facilitating further weigh loss.[97] Whether the same beneficial alterations in gut hormone composition can be achieved by diet-induced weight loss or other dietary interventions is still not clear, and promote interesting hypotheses for further research.[94]

1.4 Irritable bowel syndrome

1.4.1 Definition

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder (FGID), assumed to affect between 10 and 20% globally, with a higher prevalence reported in women than men.[98] The condition is characterized by a combination of symptoms including abdominal pain, bloating, distention, flatulence and disturbed bowel habits seen as constipation (IBS-C), diarrhea (IBS-D) or a combination of both (IBS-mixed).[99] Besides the GI symptoms, many of those suffering from IBS normally experience a broad spectrum of extra-intestinal symptoms, such as fatigue, fibromyalgia, poor social functioning and reduced emotional well-being. IBS is shown to have severe impact on the quality of life.[100,101]

1.4.2 Etiology and pathogenesis

The ethology of the disease is not fully understood, but it is evidence that the condition involve a dysfunction in one or more of the control systems that contributes to the regulation of bowel function, including the central nervous system, the enteric nervous system, the enteroendocrine system, the enteric immune system and the gut microbiota.[102,103] Suggested mechanisms includes alterations in the gut-brain axis, low-grade inflammation, visceral hypersensitivity, abnormalities in the GI endocrine cells, changes in the GI motility, post infectious changes, bacterial overgrowth, malabsorption of carbohydrates, abnormalities in serotonin metabolism, gene interactions and alterations in the gut microbiota composition.[100,104-107] An overview of suggested mechanisms and factors involved in IBS is summarized in **Figure 4.**

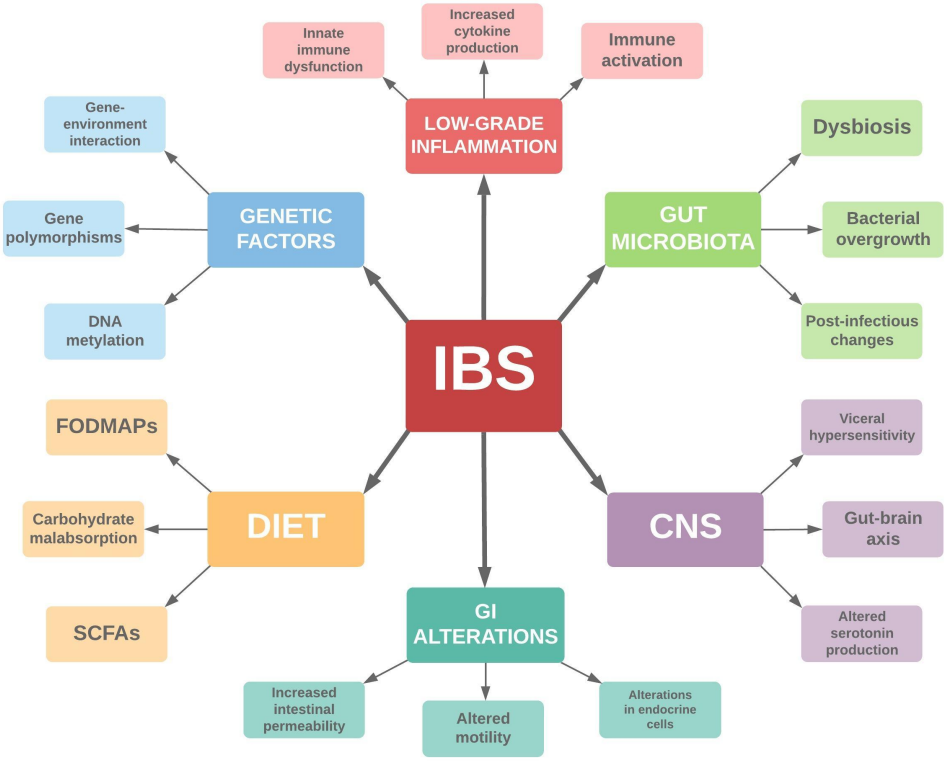


Figure 4. Overview of suggested mechanisms and factors involved in irritable bowel syndrome (IBS). CNS: Central nervous system, GI: Gastrointestinal, FODMAPs: Fermentable oligo-, di, monosaccharides and polyols, SCFAs: Short-chain fatty acids. The figure is made in Lucidchart, Dale 2019.

1.4.3 Gut microbiota and IBS

The human microbiota includes the entire collection of microorganisms living on the surface and inside our bodies. The microbiota living in the gut constitute by far the greatest portion, and it is estimated that over 70% of all the microbes in the human body is colonized in the colon, and constitute a cell mass equivalent to 1-2 kg of body weight.[108,109] Over 1000 different species have been identified in the human gut, all belonging to a small number of phyla. The most abundant phylas are *Firmicutes,*

Bacteroidetes and *Acinobacteria*, while *Proteobacteria, Fusobacteria, Cyanobacteria* and *Verrucoicrobia* are usually present in lower amounts.[110] While the gut microbiota is the present community of microorganisms, the gut microbiome refers to the entire set of genomic elements decisive for the microbiota in the gut.[111] A healthy composition of the gut microbiome is essential for a broad range of physiological functions, as the gut microbial genes is decisive for the bacterial richness in the gut and hence the activity of the gut microbiota.[110] Microbial richness, seen as bacterial diversity, is usually considered an indicator of good health. In contrast, reduced bacterial diversity and imbalance of the gut microbiota, referred to as dysbiosis, has been associated with impaired metabolism, obesity and broad range of diseases, such as T2D, immune-related diseases and inflammatory diseases, including inflammatory bowel disease (IBD).[109,112,113]

Disturbances in the gut microbiota has been increasingly linked to the pathophysiology of IBS.[114] Observations of increased risk of IBS onset after a gastroenteritis and associations between IBS and prior use of antibiotics highlights the importance of a disturbance in the gut microbiota in IBS.[115,116] In addition, a broad range of studies have reported that the gut microbiota profiles in subjects with IBS differs to the gut microbiota profiles of healthy controls.[117-120]

It is growing evidence that the altered gut microbiota in IBS not only explains the abdominal IBS symptoms, but also the psychiatric co-morbidity occurring in considerable number of patients.[121] Recent years, the interaction between diet, gut microbiota composition and IBS symptom severity has gained a lot of attention. This is currently considered an important pathophysiological basis for treatment of the condition, hence the “brain-gut” axis is lately referred to as the “brain-gut-microbiota” axis.[122]

1.4.4 Diagnosis

IBS is a functional disorder, and up to date no biomarkers have been identified to confirm the diagnosis. Thus, the diagnosis of IBS is based on clinically symptoms and consistency and frequency of stool, in addition to exclusion of other GI disorders, such as coeliac disease, IBD and microscopic colitis.[102] The diagnostic criteria for IBS has evolved since 1978, when the first criteria was published by Manning et al.[123] The changes includes the development of the diagnostic Rome guidelines, currently available in the IV edition.[99] The Rome IV criteria for diagnosis of IBS and the different subtypes are presented in **Table 2.** For an accurate diagnosis, a classification of IBS subtype according to the Bristol Stool Form Scale is recommended.[99]

Table 2. Rome IV criteria for the diagnosis of IBS. Modified from Mearin et al. 2016.[99]

Rome IV diagnostic criteria for IBS Recurrent abdominal pain on average at least 1 day per week in the last 3 months, associated with two or more of the following: 1. Related to defecation 2. Associated with a change in stool frequency 3. Associated with a change in form (appearance) of stool Symptoms must have started at least 6 months before diagnosis.	
IBS with predominant constipation (IBS-C)	25% of bowel movements with Bristol stool form types 1 or 2 AND < 25% of bowel movements with Bristol stool form types 6 or 7
IBS with predominant diarrhea (IBS-D)	>25% of bowel movements with Bristol stool form types 6 or 7 AND < 25% of bowel movements with Bristol stool form types 1 or 2
IBS with mixed bowel habits (IBS-M)	>25% of bowel movements with Bristol stool form types 1 or 2 AND > 25% of bowel movements with Bristol stool form types 6 or 7
IBS unclassified (IBS-U)	Patients who meet diagnostic criteria for IBS but whose bowel habits cannot be accurately categorized into 1 of the 3 groups above

1.4.5 Treatment

The treatment of IBS aims to target the most predominant symptoms. A central part of the treatment is to educate the patients by explaining the condition and the different treatment options, as well as provide reassurance about the symptoms as non-harmful

events.[99] Non-pharmacological treatment options are preferably the first-choice, as available drugs are most likely to target only the predominant symptom. Of note, antidiarrheals, laxatives, antispasmodics and anti-depressants are used in some cases. Currently, some of the most common drugs targeting the specific subtype-symptoms of IBS includes the guanylate cyclase C antagonist linaclotide (Constella) for patients with IBS-C and the mixed opioid agonist/antagonist eluxadoline (Truberzi) for patients with IBS-D and/or M. However, due to the broad diversity of symptoms experienced by most patients, drug treatment is often perceived as inadequate and do not lead to total control of symptoms.[99] A multidisciplinary approach is the preferred and recommended treatment option. A better understanding of the disease, combined with life-style interventions including diet, exercise and breathing techniques, is proven to be effective for reducing symptom severity in IBS patients.[100] In addition, psychological and behavioral treatment like cognitive behavioral therapy and hypnotherapy has also been shown to reduce IBS symptoms and improve quality of life.[124]

1.4.6 IBS and diet

Diet composition and food intake has been shown to play an important role in the generation of symptoms in patients diagnosed with IBS, and most patients claims that their symptoms can be related to the consumption of different foods.[125] Several modifications in the diet are shown to improve symptom severity in IBS. The first line dietary treatment for IBS patients is to follow the dietary guidelines from the modified NICE (National Institute for Health and Care Excellence) diet, as recommended by the British Dietetic Association.[126] The NICE-diet highlight the importance of regular meals, and recommend to reduce the intake of carbonated drinks, shewing gum, fatty and spicy foods, coffee, alcohol, onion, cabbage and beans as well as supplementing the diet with fibers such as psyllium husk. If these dietary modifications does not improve symptoms, a diet with a restricted intake of fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) is shown to be effective for a reduction in GI symptoms, especially abdominal pain and bloating.[127] Currently, the low-FODMAP diet is a recommended dietary treatment approach in IBS.[128]

FODMAPs are short-chained carbohydrates that are poorly absorbed in the small intestines, and when fermented by colonic bacteria, production of gas and osmotic activity causes symptoms such as bloating and diarrhea. A low-FODMAP diet is shown to significantly reduce symptoms in IBS patients.[128] Notably, a diet with a restricted intake of FODMAPs is associated with distinct alterations in both function and composition of the gut microbiota, as well as modulations in fecal fermentation and production of short chain fatty acids (SCFAs).[129,130] However, the long-term health effect of adherence to this dietary intervention is not established. The health effects of the changes observed in the gut microbiota composition and fecal fermentation in response to a diet with low content of fermentable carbohydrates is currently unknown,[131] thus the long-term use of a strict low-FODMAP diet needs to be studied further in order to explore the long term effects. Novel, additional therapeutic dietary treatments for symptom relief in IBS patients can thus arguably be valuable.

1.4.7 Potential biomarkers for IBS

Several potential biomarkers for the diagnosis of IBS have been investigated in clinical trials, including non-invasive markers in both serum and feces. Overall, the results up to date have been disappointing, and these biomarkers are currently only used in a research setting.[132] The suggested biomarkers tends to identify organic disease and hence eliminate IBS, rather than differentiate the IBS diagnosis in particular.[132]

Pro-inflammatory cytokines

All though the exact mechanisms behind IBS remains uncertain, several studies have suggested that a chronic low-grade mucosal inflammation and imbalance in the cytokine levels may contribute as potential etiological factors.[133,134] Cytokines is a collective term of signal molecules with immunological functions, affecting almost all biological processes and produced by a broad range of human cells, including mast cells, macrophages and B- and T-lymphocytes, among others. The term “cytokine” encompasses different families, and includes interleukins, chemokines, growth factors, interferons, adipokines and the tumor necrosis factor family.[135] Comparison of cytokine

levels in IBS patients compared to healthy controls have revealed altered cytokine production in IBS patients, and particularly mast cell activation has been suggested as a contributor to IBS pathogenesis.[133] A broad spectrum of different cytokines have been investigated in IBS patients, including several of the interleukins (IL), interferon-gamma (INF-γ) and TNF-α, all considered functional cytokines important for the immune system. Elevated levels of IL-6, IL-8, IL-17 and TNF-α have been reported in IBS patients compared to healthy controls,[136-138] and a decline in levels of IL-6 and IL-8 has been observed in response to a low-FODMAP intervention.[139] In addition, significantly correlations have been observed between cytokine levels, symptom severity and quality of life in IBS patiens.[138]

Gut integrity markers

In addition to low-grade inflammation, increased intestinal permeability is suggested to contribute to the IBS pathogenesis. Several markers related to the enterocyte function have been investigated in IBS populations. Zonulin is a protein serving as a physiologic modulator of intercellular tight junctions, assumed to be a master regulator on the intestinal permeability linked to development of several chronic inflammatory disorders. It is suggested that zonulin might serve as a marker for impaired gut-barrier function.[140] When investigated in IBS patients compared to healthy individuals, higher levels of zonulin was reported in patients with IBS.[141] Importantly, it was recently highlighted that serum zonulin might not be a valid marker for the evaluation of gut-mucosal barrier function in GI disorders, thus zonulin values in this group of patients should be interpreted with caution.[142]

Lipopolysaccharide-binding protein (LBP) is an acute-phase protein suggested as a marker for endotoxemia and bacterial translocation.[143] Increased levels of LBP has been reported in patients with IBD.[144] In addition, elevated concentrations has been observed in patients with non-coeliac wheat sensitivity, suggested to be a subgroup of IBS.[145] Serum LBP levels are thought to correlate with serum concentrations of lipopolysaccharide (LPS),[143] When LPS was investigated in IBS-patients, the levels were similar to those of healthy controls and did not correlate with IBS-symptoms.[146]

Intestinal fatty acid binding protein (iFABP) is considered a marker for intestinal epithelial cell damage. Lower concentrations of iFABP have been reported in IBS patients than in healthy controls.[146] Of note, these gut integrity markers related to enteric function are not specific for IBS, and up to date the implications of these markers in the diagnosis of IBS is not established.

Fecal markers

Fecal calprotectin is the most investigated fecal biomarker for inflammation. It is regarded a valid marker for inflammation in the GI tract and can be useful to discriminate between IBS and IBD.[147] Calprotectin is not related to the pathogenesis of IBS, and levels are expected to be within the normal range in patients with FGIDs. A cut-off value of 150 mg/kg has by several studies been suggested to hold good diagnostic accuracy for the distinction between IBS and IBD.[148]

Several recent studies have suggested that dysbiosis of the gut microbiota contributes to the pathogenesis of IBS, and concentrations of fecal SCFAs have been investigated as a marker of fecal fermentation reflecting the gut microbiota activity.[130] Distinct alterations in fecal SCFA concentrations have been reported between IBS patients and healthy controls, and propionate and butyrate in particular have been suggested as possible biomarkers for the distinction between IBS and healthy individuals.[149]

1.4.8 Proteins and peptides in IBS

Clinical experience indicate that dietary proteins normally are well tolerated in IBS patients, and most individuals link their symptoms to the intake of different carbohydrates.[127] Limited evidence and knowledge exists on the impact of different protein sources on gut health. It has been demonstrated in healthy individuals that different dietary sources of protein affect the diversity and composition of the gut microbiota in different degree, and thereby potentially influence different health outcomes.[150] It is growing evidence suggesting that the amino acid composition and digestibility of dietary proteins is determent for the gut microbiota composition.[151,152] Novel results indicate that the presence of fish proteins in the diet have impact on both composition and activity of the gut microbiome, affecting the human microbiota composition and activity.[153]

In addition to the results highlighting proteins and peptides as possible modulators of the gut microbiota, it has been suggested through *in vitro* models that a fish protein hydrolysate may have an immune-modulating effect with beneficial properties in the intestine.[154] A small clinical trial has reported that a dietary supplement containing hydrolyzed proteins from pacific whiting may prevent NSAID-induced injuries in the gut.[67,69] Despite the lack of research supporting an effect of supplements with peptides and protein hydrolysates on gut health, several dietary supplements containing peptides are today sold as commercial products targeting IBS patients and those suffering from digestive problems (e.g. Peptid+® and TRP™).

1.5 Rationale and hypothesis

As described in the scientific background, emerging evidence suggest that fish protein hydrolysates may have several beneficial health effects, also when supplemented in low doses compared to the normal daily dietary protein intake. The prevalence of overweight and obesity is constantly increasing worldwide, and lifestyle related diseases leading to impaired glucose metabolism and other adverse metabolic impairments, with the need of treatment and prevention strategies, affect a huge part of the world's population. In addition, around one out of ten individuals suffers from IBS symptoms, a diagnosis only partly controlled by alterations in the diet and/or pharmacological treatment, with the need of additional treatment strategies. A dietary supplement contributing to increased utilization of residual material from the fishing industry, capable of improve health outcomes related to metabolism and GI function, can thus arguably be valuable both for human health and the industry.

Overall, we hypothesized that a supplement with cod protein hydrolysate (CPH) would beneficially affect health by improve outcomes related to glucose metabolism, appetite and gut health. More specific, we hypothesized that:

i) A low dose of supplement with CPH would:

- beneficially affect glucose metabolism, by lowering the postprandial glucose and insulin concentrations in healthy subjects **(Paper I)**
- reduce postprandial concentrations of the appetite regulating hormone ghrelin, hence reduce the feeling of hunger in healthy subjects **(Paper II)**
- beneficially affect gut health and improve symptoms in IBS patients, by reduce degree gut permeability and inflammation, as well as modulate the composition of the gut microbiota and hence the fecal concentration of short chain fatty acids (SCFAs) **(Paper IV)**

ii) It was a dose-response relationship between different doses of CPH supplementation and degree of changes in the glucose metabolism in older adults **(Paper III)**

2. Objectives

The overall aim of the project was to investigate whether CPH produced from hydrolysis of residual material (muscle) from cod filet production have beneficial properties in the human body, leading to improvement of health outcomes related to glucose regulation and appetite in healthy subjects, and gut health in patients with IBS. The work presented in the current thesis is based on an initiative from Firmenich Bjørge Biomarin AS, Ålesund, Norway, manufacturer of the specific CPH investigated in this PhD-project.

Three intervention studies were included to address the effects of the CPH. Two intervention studies were carried out in healthy subjects to investigate the effect of CPH supplementation on postprandial glucose metabolism and appetite and identify the most effective dose for further use. The last study was designed to evaluate the effect of a CPH supplement in patients with IBS. The specific aims of the three studies and the four papers included in this PhD-thesis are listed below:

Study 1 (Paper I and II):

The primary aim was to assess the effect of a single, low dose of CPH on postprandial glucose metabolism in healthy adults (Paper I). The secondary aim was to evaluate the effect on outcomes related to appetite (Paper II).

Study 2 (Paper III):

The aim was to investigate the dose-response of increasing doses of CPH supplementation on glucose metabolism in older adults, to find a potential effective daily dose for further use in clinical studies.

Study 3 (Paper IV):

The aim was to investigate the effect of a CPH supplement on gut health in patients with IBS, by evaluating symptom severity, gut integrity markers and pro-inflammatory cytokines in serum, fecal calprotectin and fecal fermentation measured by concentration of short-chain fatty acids (SCFAs).

3. Methods

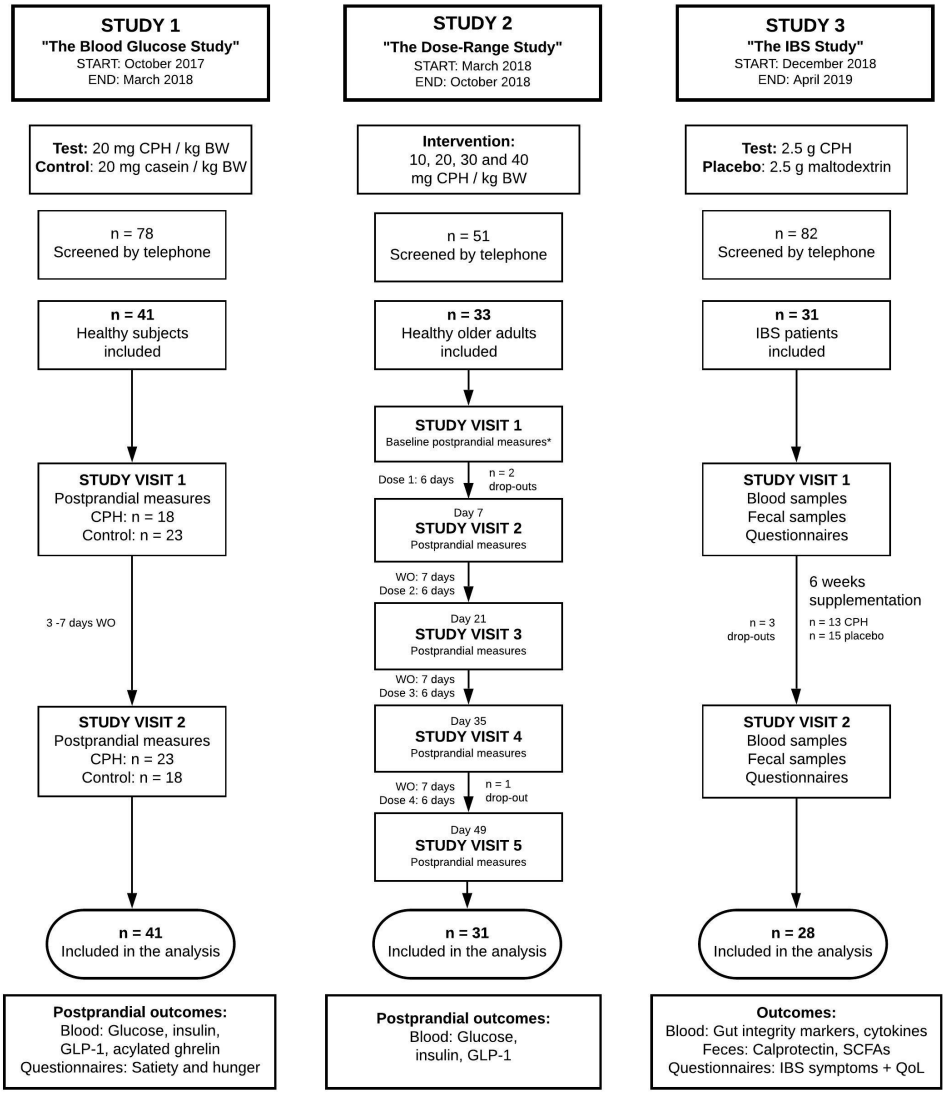
Methods for the three clinical studies conducted in the work with this thesis are described below. For a detailed description of methods used for laboratory analysis of the outcome measures, composition of the test material used in the three different studies and the implemented questionnaires, the reader is referred to the respective papers.

3.1 Study population and design

An overview of the recruitment flow, study design and study outcomes for the three randomized controlled trials included in this PhD-project is shown in **Figure 5.** Paper I and II are based on Study 1, Paper III is based on Study 2 and Paper IV is based on Study 3.

3.1.1 Paper I and II

Participants were recruited through advertisements on the internet and posters at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018. Interested participants were contacted by telephone for evaluation of eligibility and compliance with inclusion criteria. Suitable candidates were invited to further screening at a hospital visit. Forty-one healthy individuals were included in the trial, of whom 26 were females and 15 males. Inclusion criteria were age 40-65 years and BMI 20-30 kg/m^2. Exclusion criteria were fish/shellfish allergy and intolerance, pharmacologically treated diabetes mellitus, elevated blood pressure, chronic diseases that might affect the evaluation of the study endpoints and acute infections. Participants were instructed to avoid omega-3 supplementation 1 week prior to study start and while participating in the study, and not make any changes in their diet or level of physical activity. The study was designed as a double-blind crossover trial and included two different study days, with three to seven days wash-out period in between. On both study days the participants were served a test drink containing either 20 mg of CPH per kg body weight or casein given as control. The test material was delivered in neutral bottles, containing a lemon-flavored powder to be mixed with water.



CPH: Cod protein hydrolysate, BW: Body weight, WO: Wash out, QoL: Quality of life
*Results from study visit 1 with baseline postprandial measures in Study 2 is not included in the results reported in the manuscript based on the study (Paper III). See explanation in paragraph 6.3.2.

Figure 5. Overview of the recruitment flow, study design and study outcomes for the three randomized controlled trials included in the current PhD-project. Paper I and II are based on Study 1, Paper III is based on Study 2 and Paper IV is based on Study 3.

On both study days the participants met in a fasting state at the hospital between 8 and 9 am. Baseline blood samples were taken before they were served the test drink (CPH or control in random order) and a breakfast meal. The breakfast including the test drink provided 500 kcal and 77 g carbohydrate. Blood samples were taken in intervals of 20 minutes until 120 minutes postprandially, with a final sample after 180 minutes.

The primary outcome was response in postprandial glucose metabolism, measured by venous samples of serum glucose and insulin, and plasma GLP-1 (Paper I). Secondary outcomes were postprandial acylated ghrelin concentrations and sensations associated with appetite (satiety and the feeling of fullness) measured by symptom questionnaires (Paper II).

3.1.2 Paper III

Participants were recruited by advertisements online and at Haukeland University Hospital and Ålesund Hospital between March and July 2018. Subjects interested in participating were contacted and screened for eligibility by telephone. Suitable candidates meeting the inclusion criteria were invited to a baseline hospital visit including baseline blood chemistry and signing of the informed consent form. The criteria for inclusion were age between 60 and 80 years, BMI between 20-30 kg/m^2 and signed informed consent. Criteria for exclusion were allergy and intolerances to fish and/or shellfish, pharmacologically treated diabetes mellitus, low and/or unstable blood pressure, chronic diseases or medication that were likely to interfere with the evaluation of the study endpoints, acute infections, substance misuse or unwillingness to comply with the requirements of the study. The participants were instructed not to take any nutritional supplements containing omega-3 fatty acids for two weeks before study commencement, and during the course of the study.

In addition to the baseline meeting, the participants attended five study visits (Figure 5). The first study visit included baseline postprandial measures before the participants had taken any doses of CPH. These results are not reported in the manuscript according to a

methodological error, see further descriptions under discussion of results (paragraph 6.3.2). The study included four different intervention cycles of one week each, with doses corresponding to 10, 20, 30 and 40 mg per kg body weight CPH supplement provided in randomized order. The participants received six bottles with powder to be mixed with water (test drink), to be taken each morning. On day 7 in each intervention period, the participants met in a fasting state at the hospital between 8 and 9 am. The last dose of the current intervention period (test drink) was given when fasting baseline blood samples were taken. Breakfast was served 10 minutes after the test drink. The breakfast including the test drink provided 455 kcal and 63.5 g carbohydrate. Blood samples were taken at 20 minutes intervals until 120 minutes postprandially, with a final sample after 180 minutes. The participants received six bottles for the next intervention week at the end of each study day. It was a wash-out period of seven days between each intervention week. Participants received reminders on text messages prior to each new intervention week, and before each study day.

The primary outcome was postprandial response in glucose metabolism after the four different doses, measured by venous samples of serum glucose and insulin, and plasma GLP-1.

3.1.3 Paper IV

IBS patients were recruited through online advertisement including a digital recruitment form, between December 2018 and January 2019. Suitable candidates meeting the inclusion criteria were contacted and screened over telephone. After receiving information about the study and signing the informed consent form, all patients received a dietary record and equipment to collect a baseline stool sample by mail, prior to the first hospital visit. Inclusion criteria were age 20-70 years, BMI 18-30 kg/m^2 and IBS diagnosis according to Rome IV criteria with predominant diarrhea (IBS-D) or mixed bowel movements (IBS-M). Exclusion criteria were fish/shellfish allergy and intolerance, diabetes mellitus, elevated blood pressure, chronic diseases that might affect the evaluation of the study outcomes, acute infections, substance abuse,

immunocompromised patients defined as taking immuno-suppressive medications, patients eating a strict low-FODMAP diet, use of antibiotics the last 4 weeks before inclusion or use of medications for the IBS diagnosis.

The study was a double-blinded, randomized parallel group trial, and included a six-week intervention with a drink containing 2.5 g CPH (test material) or 2.5 g maltodextrin (placebo). Medical data, the IBS-diagnosis and biochemical variables were assessed at baseline. The Rome IV criteria were used to confirm the clinical diagnosis of IBS.

A 3-day dietary record was completed by all patients prior to taking the fecal sample at baseline and at end of the study. The patients were instructed not to make any changes in the diet while attending the study, and not to take any nutritional supplements containing omega-3 or pre- or probiotics for 6 weeks before study start, and during the trial.

Outcomes were evaluated at baseline and at end of the study (after six weeks). Primary outcomes were symptom severity evaluated by IBS Severity Scoring System (IBS-SSS) and quality of life. Secondary outcomes included gut integrity markers (zonulin, LBP, iFABP) and pro-inflammatory cytokines in serum (TNF-α , INF-γ, IL-4, IL-6, IL-8, IL-10), fecal calprotectin and fecal fermentation measured by concentration of SCFAs.

3.2 Test material: The cod protein hydrolysate

The fish protein hydrolysate investigated in this PhD-project was manufactured by Firmenich Bjørge Biomarin AS, Ålesund, Norway. A powder was made by hydrolysing fish muscle of Atlantic cod from the production of cod filets (cutting left overs). Fish muscle was hydrolysed with Protamex® (Novozymes AS) followed by spray drying of the soluble part of the enzyme digest.

The CPH raw material contained approximately 89% protein by weight, as well as <0.2% fat, 0% carbohydrate, <3.0% water, 10% ash, 0.1% NaCl, 1.7% sodium and 0.07% chloride. Free amino acids accounted for 4.77 % of total amino acids in the CPH, and the ratio essential amino acids/non-essential amino acids was 0.70. Analysis of the molecular weight distribution showed that about 90% of the peptides in the hydrolysate had a molecular weight of 2000 Daltons (Da) or less (18 amino acids or less), about 75% of 1000 Da or less (10 amino acids or less) while about 55% had a molecular weight of 500 Da or less (5 amino acids or less). Approximately 25 to 30% of the peptides had a molecular weight less than 200 Da, which represented small dipeptides and free amino acids (data obtained from Firmenich Bjørge Biomarin AS, Ålesund, Norway).

The CPH was analysed for allergenicity, and it was found to be so low that it was regarded safe for those with fish allergy to consume (Report from Firmenich Bjørge Biomarin AS, analysis performed by the Section for Protein and Allergy Analysis, Laboratory for Clinical Biochemistry, Haukeland University Hospital, data on file).

The amino acid and taurine composition of the CPH used in all of the three clinical studies included in this PhD-project is shown in **Table 3**. Composition of the different test materials used in the three different interventions, all provided as powders to be mixed with water, are described in detail in each of the corresponding papers.

Table 3. Amino acid and taurine composition of the cod protein hydrolysate (CPH) used in the three clinical studies included in this PhD-thesis.

Amino acid	Total amino acid (mg/g)
Alanine	47.8
Arginine	51.1
Aspartic acid	73.3
Aspargine	0.38
Glutamatic acid	125.0
Glutamine	0.78
Glycine	50.9
Histidine	13.5
Hydroxyproline	1.0
Isoleucine*	30.1
Leucine*	60.3
Lysine	71.3
Methionine	22.1
Phenylalanine	23.2
Proline	29.7
Serine	36.0
Taurine	6.6
Threonine	30.9
Thryptophan	6.0
Tyrosine	22.7
Valine*	36.9

*Branched-chained amino acids (BCAAs)

3.3 Statistical analyses

SPSS data package (IBM SPSS Statistics 24), Stata v15.1 (StataCorp LLC, Texas, USA) and GraphPad Prism version 7.0 (GraphPad Software, Inc., San Diego, CA, USA) were used for the statistical analysis included in the current thesis. All graphical work was conducted in GraphPad. P-values <0.05 were considered statistically significant.

3.3.1 Paper I and II

A multivariable, repeated measures linear mixed-effects regression analysis was conducted to evaluate the difference between the CPH and control arms. The data for insulin, GLP-1 and acylated ghrelin was not normally distributed, thus it was log-transformed before analysis. The analyses were adjusted for BMI and gender. Total area under the curve (tAUC) for postprandial glucose concentration, differences in baseline characteristics between genders, acylated ghrelin and postprandial scores for satiety and the feeling of fullness were compared using unpaired t-tests. Paired t-test was used to evaluate the differences in nutrient intake between the two study days.

3.3.2 Paper III

To estimate the effect of the dose, the maximum observed value and the AUC of each outcome variable for each combination of person and dose was calculated. Mixed effects models were fitted with the outcome measure (max value or AUC) as dependent variable, fixed effects of dose and random intercepts across persons. Potential carry-over effects were assessed using a standard likelihood-ratio test, to test for interaction between ordering and dose.

3.3.3 Paper IV

Paired two-sided t-tests were used to compare differences between the baseline and after intervention measures for each group, whereas unpaired two-sided t-tests were used to compare differences between the CPH group and the placebo group. Correlations were assessed with Pearson´s correlation coefficient.

3.4 Ethics

All the clinical studies were conducted according to the guidelines laid down in the Declaration of Helsinki and the Regional Committee for Medical and Health Research Ethics of Central Norway approved all procedures involving human subjects. Written informed consent was obtained from all subjects.

Paper I and II: The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2017/1794). The trial was registered at clinicaltrials.gov as NCT03669796.

Paper III: The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2017/1795). The trial was registered at clinicaltrials.gov as NCT03526744.

Paper IV: The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2018/1825). The trial was registered at clinicaltrials.gov as NCT03801057.

4. Results

A summary of the most important findings in each paper is presented in the following sections.

4.1 Paper I

Forty-one participants (age 51±6 years), of whom twenty-six females, completed the trial. Mean energy intake before the two study visits did not differ and mean fasting blood glucose concentrations were equal on both study days. At the first study visit, eighteen participants were allocated to CPH and twenty-three were allocated to control. At the second study visit they were all allocated to the opposite intervention to the one received the first study day.

The peak in glucose concentration occurred 20 minutes after the breakfast both after CPH and control, and was numerically higher after CPH drink than control, however did not differ significantly. After CPH, the insulin concentration peaked at a lower level and after 20 minutes, whereas the peak after control was higher and occurred 40 minutes postprandially. The insulin concentration was significantly lower after CPH supplementation than after control. Mean plasma GPL-1 concentrations were equal before each study day, and the concentrations dropped right after the meal, with lower concentrations observed after CPH than after control. No differences between CPH supplement and control was observed for postprandial concentrations of GLP-1.

4.2 Paper II

The included participants and allocation to supplement is similar to that presented for Paper 1. No significant differences were observed between CPH and control for acylated ghrelin concentrations postprandially in a mixed-effects regression analysis. No effects were seen for BMI or gender when adjusting for these parameters in the analysis. Mean fasting acylated ghrelin levels were comparable before the CPH intervention and the control intervention. The concentration of acylated ghrelin was lowest 80 minutes postprandially after CPH and 20 minutes postprandially after control. No correlation was observed between body weight (kg) and concentrations of acylated ghrelin at baseline. When comparing the tAUC for postprandial acylated ghrelin and postprandial scores for satiety and the feeling of fullness, no differences were observed between CPH and control.

4.3 Paper III

Thirty-three older adults were included, of whom 31 subjects (18 females) completed the study (age 67.8±4.9 years) and were included in the analysis. Energy and macronutrient intake did not change during the study period. No differences in estimated maximum levels of glucose, insulin or GLP-1 were observed when comparing the lowest dose of CPH (10 mg/kg body weight) to the higher doses (20, 30 or 40 mg/kg body weight). Estimated maximum value of glucose was on average 0.28 mmol/L lower when the participants had been given the highest dose (40 mg/kg body weight CPH) compared to the lowest dose (10 mg/kg body weight CPH). Estimated maximum level of insulin was on average 5.14 mIU/L lower when the participants had been given the highest dose compared to the lowest dose. Estimated maximum level of GLP-1 was on average 0.34 pmol/l lower when given the highest dose compared to the lowest dose. No statistically significant differences in AUC between the four different doses were observed for either glucose, insulin or GLP-1 when comparing the lowest dose of 10 mg/kg body weight of CPH with the higher doses of 20, 30 or 40 mg/kg body weight. No carry-over effect was observed for any of the outcome measures.

4.4 Paper IV

Thirty-one IBS patients were included of whom 28 patients (23 women and 5 men) completed the trial (age 42.7±11.9 years). Three patients withdrew after randomization, one patient due to disliking the supplement and two patients due to experiencing increased diarrhoea. Thirteen patients were allocated to CPH supplementation whereas 15 patients received placebo. The groups were comparable at baseline. Total IBS-SSS scores and scores for quality of life were reduced in both the CPH group and the placebo-group in response to intervention, with a significant reduction only in the placebo group. End of study scores did not differ between groups. Concentrations of gut integrity markers, pro-inflammatory cytokines and SCFAs did not change in response to intervention for any of the groups. Baseline measures for the whole group showed that the total SCFA concentration was significantly inversely correlated with the total IBS-SSS score. No correlations were found between baseline total IBS-SSS score or any of the serum markers.

5. Methodological considerations

5.1 The cod protein hydrolysate

5.1.1 Rationale for choice of doses

The scientific rationale behind the choice of amount of CPH (20 mg/kg body weight), administered in Study 1 (Paper I and II) is limited. Unpublished data from studies with the CPH performed in rats and a small number of human subjects have indicated that the hydrolysate may induce a metabolic response when given in low concentrations. A cross-over trial including 12 healthy males found premeal supplementation with 20 mg/kg body weight CPH to reduce postprandial blood glucose and insulin concentrations when compared to casein and whey protein. In addition, a study in 9 healthy subjects found one premeal supplementation with 20 mg/kg body weight CPH to increase postprandial secretion of GLP-1 and PYY, reduce the postprandial secretion of ghrelin as well as reduce the feeling of hunger and increase the feeling of fullness after the test meal according to included questionnaires (unpublished data from studies performed at Clinical Trial Consultants AB, University Hospital, Uppsala University, Sweden, provided by Einar Lied, former scientific advisor for Firmenich Bjørge Biomarin AS).

Of note, these unpublished trials included few subjects and the tendencies reported were not analyzed according to statistical significance. Anyhow, they suggested a metabolic effect when the CPH was given in concentrations of 20 mg/kg body weight, hence this dose was chosen as a starting point of investigation in Study 1.

Due to limited knowledge and few published papers on the effect of fish protein hydrolysates in the human body, we consider Study 1 to be an explorative trial regarding the unique effect of such a low dose. Based on the previously described literature reporting on the effect of fish protein hydrolysates in human subjects, the suggested effective daily dose ranges from 1 to 6 g a day.[61,63,64] Taken together, results from these studies support the hypothesis that supplementation with a low dose of cod protein

hydrolysate may beneficially affect glucose metabolism and hormones related to appetite and suggests that bioactivity from fish protein hydrolysates may be obtained at supplementary dosages of around 1.5 g a day, equivalent to about 15-20 mg hydrolysate per kg body weight in an individual with a body weight of 70-80 kg.

Of note, the specific mechanisms behind the reported effects are not known. However, we chose to further investigate the potential effect of low doses of CPH in both the following interventions (Study 2 and 3). As the results from Paper III (the dose-range study) was not ready during the planning of the third study in IBS patients, we found it appropriate to decide on a set dose of 2.5 g of CPH a day for all individuals included. Overall, it is possible that the different doses investigated in the studies included in the current PhD-project were too low to observe an effect, and that the low doses investigated might partly explain the lack of distinct effects.

5.1.2 Hypothesized mechanistic effects

The recommended lowest daily dietary protein intake of a healthy individual is 0.8-1.0 g protein per kg body weight per day,[42] equal to 65-80 g protein per day with a body weight of 80 kg and on average e.g. 20 g protein per meal. Consequently, the hypothesized effect of the CPH is not due to the added consumed protein *per se,* which is negligible compared to the average daily protein intake. The mechanism of action of any effects of the CPH investigated in this project is still unclear. A low dose of fish protein hydrolysate is presumed to be effective due to the content of bioactive peptides with unique amino acid sequences, possibly capable of rapid absorption and distribution in the body,[44] however the CPH investigated in the current project is not analyzed for the content of bioactive peptides.

We postulated that the hydrolysate investigated in the project would have an effect beyond being a source of amino acids and protein, and that a possible effect of this specific hydrolysate could be attributed to the high content of branched chained amino acids (BCAAs) present as di- or tripeptides. BCAAs act as signal molecules affecting

protein synthesis, glucose metabolism and regulation of body weigh through pathways such as the PI3K/Akt and mTOR pathway.[155] High levels of circulating BCAAs are suggested to be closely related to several adverse metabolic outcomes, and increasing evidence suggests that insulin resistance, obesity and T2D can be linked to dysregulation of the BCAA metabolism, resulting in elevated circulating levels of these.[156,157] Hence, high levels of circulating BCAAs have been found to serve as biomarkers for insulin resistance and risk of developing T2D, and pharmacological treatment strategies have aimed to target the dysregulated signaling network including the BCAAs, such as the PI3K/Akt and mTOR pathway.[156]

Although recent studies have indicated that the circulating levels of BCAAs are elevated in obese and insulin-resistant individuals, the literature is currently unclear regards to whether supplementation with additional BCAAs in the diet will further impair the glucose metabolism. Both animal and human trials have demonstrated that dietary BCAAs may contribute to impaired glucose regulation.[158,159] However, this is not consistent, and a large Japanese cohort study suggested that a high intake of dietary BCAAs may be associated with a decreased risk of diabetes.[160] In addition, a recent study in twelve obese and prediabetic individuals reported that 4 weeks of supplementation with 20 g BCAA a day did not impair glucose metabolism.[161] Also, it has previously been demonstrated that postprandial plasma levels of BCAAs can be correlated with a beneficial insulin response and the release of the gut hormones GLP-1 and GIP after a meal with different protein sourses.[162] Interestingly, intragastric administration of the BCAAs leucine and isoleucine before a mixed nutrient drink has been demonstrated to reduce blood glucose response postprandially in healthy subjects.[163] In addition, cell studies have suggested that protein hydrolysates containing BCAAs present as mixed dipeptides in low concentrations stimulate glucose uptake in muscle cells by the PI3K/Akt pathway.[163,164] Taken together, these results suggests that dietary supplementation with a low dose of a protein hydrolysate containing BCAAs, preferably as dipeptides, might be capable of beneficially affecting glucose metabolism.

The CPH investigated in the current project had a high concentration of BCAAs, and about 10% of the di- and tripeptide fraction of the supplement was present as peptides containing leucine and/or isoleucine (**Table 3**). In summary, we postulate that these peptides might constitute a unique, bioactive effect even when given in low concentrations.

5.1.3 Interventions with food versus supplements

The present thesis investigated the effect of a dietary supplement on several different metabolic outcomes. Overall, it is much easier to conduct an intervention study with a supplement than with actual food, both according to practical and scientific reasons. A dietary supplement can be taken without changing the baseline diet, meaning that if an effect was to be observed, it is more likely to be attributed to the intervention material. When using whole foods in an intervention study, for instance lean fish to investigate the effect of fish proteins, several issues have to be considered when interpreting the results, such as bioavailability of the amino acids in the food, other nutrients in the food and possible nutrient interactions, change of energy intake and distribution of macronutrients due to the intervention. As an example, in interventions with lean fish, one has to acknowledge that the fish provided is eaten instead of another food, in many cases most likely meat. This adds an uncertainty to the design, as one can question if an effect then can be related to consumption of lean fish or rather just a reduction in meat intake. These factors, leading to a potentially biased result, is easier to standardize when the intervention consists only of a dietary supplement, not leading to a change in the normal food-, energy- and macronutrient intake, but simply adds a new factor on top.

5.2 Choice of control material

5.2.1 Study 1

In Study 1, whole casein was chosen as control ingredient to facilitate an iso-caloric and iso-nitrogenic control material. The use of casein as control can be a limitation to the design. The metabolic response to casein is well investigated, and casein is reported to be a slowly digestible protein capable of delaying gastric emptying.[165,166] In addition, the digestion pattern of casein has been suggested to hold distinct differences from other proteins when investigated in animals.[150,167,168] Casein has been shown to affect gut hormones involved in the glucose regulation as well as affect the absorption rate of different amino acids, when compared to other food proteins in healthy subjects.[162] Importantly, the most distinct differences between casein and other proteins has been reported when casein is investigated in high-protein diets as well as served as a hydrolysate,[150,167,168] which was not the case in the current trial.

One could argue that the control drink should have been a true placebo and only contained glucose. However, then it would have been possible that an observed effect would simply occur due to the differences in caloric or nitrogen content, and not to the effect of the CPH. Both test drinks contained equal amounts of protein, in total approximately 1.5 g (dependent on the participants´ body weight). This amount of protein is negligible when compared to the effect of the proteins given in the breakfast meal (12.5 g), thus a potential effect can possibly be attributed the content of bioactive peptides and not the protein *per se*.

Whole casein was chosen as control because it has been proven not to affect the glucose metabolism when administered in low concentrations.[165,169] Based on the issues described above, one might think of casein as a “positive” control.

5.2.2 Study 2

The protocol for Study 2 originally included a baseline test day without CPH supplement. Here, a glass of orange juice was chosen as control to facilitate an iso-caloric breakfast each test day, and because we aimed to investigate the effect of different doses of CPH compared to no protein. As described in detail in section 6.3.2 the baseline test day with orange juice as control was not included in the manuscript, according to a methodological mistake. Of note, a maltodextrin powder identical to the implemented powder-doses, but without CPH, could arguably be a more proper and comparable control than orange juice, and should have been implemented if one were to choose control material again.

5.2.3 Study 3

The placebo material used in Study 3 was maltodextrin. Maltodextrin is an easily absorbed polysaccharide, often used as a sugar replacement. It is thought to be a well-tolerated carbohydrate in IBS and is commonly used as placebo material in dietary interventions including IBS patients, hence can be considered a safe placebo material.[170,171] As we aimed to investigate the effect of CPH compared to a control without protein (as it was no need for an iso-nitrogenic control), we found maltodextrin to be a successful placebo material in the current trial.

5.3 The test meals

Study 1 and 2 included a meal tolerance test with serving of a standardized breakfast meal (described in section 3.1.1 and 3.1.2), to investigate changes in postprandial blood glucose metabolism. A meal tolerance test is not a standardized tool and can vary greatly in terms of nutrient composition and total energy content. A standardized test to evaluate blood glucose response is the oral glucose tolerance test (OGTT), consisting of 75 g glucose resolved in water. This method is frequently used in clinical trials when investigating postprandial glucose regulation and is a recommended test in the diagnosis of diabetes mellitus.[172] However, it is suggested that a mixed meal with proper food might be a more physiologically relevant test than an OGTT for postprandial measures, as it provides a mix of macronutrients, including both proteins and fats, which is likely to influence the glucose regulation.[173,174]

We chose to use a "normal Norwegian breakfast" consisting of two semi-dark slices of bread with butter, cheese and jam, as we wanted to investigate the metabolic effect of CPH after consumption of a regular meal with complex sources of both carbohydrates, fat and protein (representative for a normal everyday breakfast), and not only glucose. Other studies investigating effects of fish proteins on glucose regulation have implemented similar meals to the one we included.[34,39,47] As the test drinks differed slightly in composition between Study 1 and Study 2, the total content of carbohydrate was 77 g and 63,5 g, respectively. The use of the same meal in both Study 1 and 2 can arguably be regarded a strength and make the results more valid for comparison.

In line with the body-weigh-adjusted doses of CPH, one could argue that individually weight-adjusted meals, with a set distribution of energy and nutrients according to body weight, should have been implemented to strengthen the design. This was evaluated as an option when planning the study. However, we chose not to implement these individual adjustments in the breakfast meal, as it would have made the practical implementation of the study days and preparation of the breakfast meals too time consuming.

5.4 Study populations

5.4.1 Healthy middle-aged to elderly adults (Study 1 and 2)

Study 1 and 2 were designed to investigate the effect of the CPH on postprandial blood glucose metabolism. In Study 1 and 2 we chose to include healthy individuals with a BMI from 20-30 kg/m^2, corresponding to normal weight to overweight. The rationale for including healthy individuals with a BMI corresponding to normal weight to overweight, and not obesity, was the aim of primarily investigate the effect in subjects supposed to have a normal blood glucose regulation. Hence, we did not expect a great effect on the postprandial blood glucose levels, as this is supposed to be adequately regulated in a healthy individual. But, if an effect was to be observed in other outcomes in healthy individuals, it could arguably be an effect more distinct if investigated further in individuals with an abnormal blood glucose response, such as metabolic syndrome or T2D.

Previous studies investigating the effect of fish protein hydrolysate supplements on blood glucose metabolism have included healthy overweigh and obese individuals with a BMI (mean/median) range from 31-35 kg/m^2.[47,61,62] Although these individuals were characterized as "healthy overweight and/or obese", they may arguably be more likely to have a slightly dysregulated blood glucose metabolism than those subjects included in our trial.

In study 1, we included individuals aged 40-65 years, as it was a study in "normal adults" to establish a potential effect. In study 2 we included healthy individuals aged 60 to 80 years. The glucose metabolism changes with increasing age, and many elderly adults are affected by impaired glucose metabolism.[175] The skeletal muscle is the major site of insulin-stimulated glucose uptake in the human body, and aging leads to a gradual decline in muscle mass.[176] Therefore, we found it relevant to explore the potential favourable effects of CPH on parameters of glucose metabolism, as these are closely related to muscle health, in an older population.

5.4.2 IBS patients (Study 3)

Study 3 was designed to investigate the effect of a CPH supplement on gut health in patients with IBS. Patients with predominantly diarrhea or mixed bowel habits (IBS-D or IBS-M) were chosen, as these subgroups previously have been reported to benefit the most from dietary interventions, such as reported for the low-FODMAP diet.[177] In addition, by restricting the inclusion of IBS-patients to these two subgroups and not include patients with constipation as the predominant symptom, we aimed to reduce the risk of selection bias by comparing individuals with similar symptomatic problems. In addition, levels of SCFAs are suggested to differ between IBS-D/M and IBS-C,[178] hence the inclusion of patients with constipation-predominant IBS could potentially have affected this outcome. However, it can be argued that inclusion of all subtypes of IBS might could have strengthen the design, as the effect of different dietary interventions comparing all subgroups of IBS is not clearly charted out according to existing literature.

5.5 Estimation of sample size

The number of participants to include in the three clinical trials included in this PhD-project was not calculated according to a power analysis, due to lack of similar studies and knowledge on which effects to expect. Available studies investigating the effects of cod proteins in humans, published before the planning of the study presented in Paper I and II, are based on whole fish[38] or long-term use of fish protein supplement.[47,61] Thus, we had no suitable data as basis for a power analysis according to our study design. Accordingly, it is possible that too few participants to be able to observe an effect were included in the studies. We decided to include between 30 and 40 subjects in the trials, a number higher or similar to previously reported in studies on cod protein.[38,47,61] In the last trial in IBS patients we intended to include 30 patients according to protocol. To our knowledge, no previous trials have reported on the effect of a cod peptide supplement in an IBS population. Hence, we did not have adequate data to perform a power analysis.

5.6 Fecal fermentation (Study 3)

One of the secondary outcomes of Study 3 was concentration of SCFAs in feces. SCFAs are produced by microbial fermentation in the colon and serve as the main source of energy for the colonocytes. In addition, the SCFAs hold a broad spectrum of diverse physiological functions, such as influence on the colonic blood flow and the intestinal pH, of which the latter influence the absorption of nutrients.[179] SCFAs are also suggested to serve as mediators in the microbiota-gut-brain crosstalk, hence influence psychological functioning.[122]

The primary source for colonic production of SCFAs by the gut microbiota is dietary fibres. It is well established that the presence and production of SCFAs in the colon is important for health, and that the gut microbiota composition affects the production of SCFAs.[122] Although most of the SCFAs are absorbed by the colonocytes in the proximal colon and only a small fraction is left in feces, several studies have suggested that the SCFAs present in the distal gut may contribute to the regulation of motility and sensitivity,[180] as well as modulate inflammation.[181] Of note, the concentration of SCFAs in feces might also be affected by transit time, and concentrations of the major SCFAs in feces are suggested to be higher in patients with IBS-D compared to other IBS subtypes.[130]

A dietary FODMAP restriction has previously been shown to significantly modulate fecal fermentation and composition of SCFAs in patients with IBS. A 4-week low FODMAP diet in sixty-three IBS patients reported reduced concentration of total SCFAs after the diet, in addition to reduced concentrations of butyric acid and acetic acid.[130] These findings are supported by an intervention in 20 IBS patients, who found a low-FODMAP diet to significantly reduce the total concentration of SCFAs as well as butyrate.[139] In addition, a recent meta-analysis investigating the implication of alterations in fecal SCFAs in IBS patients suggested that the levels of butyrate and propionate may serve as possible biomarkers for the diagnosis of IBS.[149]

Of note, the clinical relevance and importance of changes in levels of SCFAs is currently not known.[130]

In accordance with the previously reported effect of a low-FODMAP diet on fecal fermentation and concentrations of SCFAs in IBS patients, we chose to include analysis of fecal SCFAs concentrations in our study including IBS patients. We hypothesised that if the CPH had an effect on the function and composition of the gut microbiota, the concentrations of SCFAs would serve as a marker for this. The carbohydrate intake of the participants was not supposed to change during the trial, hence if a change in SCFAs was to be observed we hypothesized that it could potentially be attributed to alterations in the gut microbiota function and composition in response to the 6-weeks with CPH supplementation.

6. Discussion of results

The overall aim of the current thesis was to investigate the potential health effects of a dietary supplement with a protein hydrolysate from cod. Effects on glucose metabolism and appetite in healthy individuals and gut health in patients with IBS were the primary outcomes. Three intervention studies were conducted in this thesis to investigate the effect of CPH supplementation on glucose metabolism (Paper I) and appetite (Paper II), the effect of increasing doses of CPH supplementation on glucose metabolism (Paper III) as well as the effect of CPH supplementation in IBS patients (Paper IV). In summary, our findings suggests that a low dose of CPH beneficially affect glucose metabolism by decreasing the postprandial insulin concentration in healthy adults (Paper I), and that a beneficial postprandial effect may seem to be more distinct if the supplement is given in a dose corresponding to 40 mg/kg body weight, compared to lower doses (Paper III). No distinct effects of CPH supplementation were observed for outcomes related to appetite regulation or gut health in IBS patients (Paper II and IV).

Previous studies have investigated the effect of lean fish and cod protein consumption in humans, as well as the effect of fish protein hydrolysates on several metabolic markers in animal models, but only a small amount of data exists on the specific effect of different fish protein hydrolysates on human metabolism. Thus, results from the presented studies add knowledge to the effects of CPH as a dietary supplement in humans and can be regarded valuable in further investigations of the effect of potentially bioactive fish peptides in individuals with abnormal glucose metabolism as well as the effect of peptides in patients with IBS. Of note, the effect of a supplement with fish peptides, or peptides from other protein sources, in an IBS population, has to our knowledge not previously been investigated. Hence, this study can be considered valuable as basis for further research on the effect of peptides in IBS, despite the lack of distinct findings.

In the following section, the main finding in the four papers as well as limitations to the design not highlighted in depth in the papers, will be discussed.

6.1 Effects on glucose metabolism (Paper I)

Paper I is based on the primary outcomes in Study 1; The effect of CPH supplementation on postprandial glucose metabolism. We reported that the postprandial insulin concentration was significantly lower after a single preprandial intake of CPH compared to control (casein), whereas no effect was observed for concentrations of glucose or GLP-1.

The trial included healthy normal- to overweight subjects with HbA1c levels within the normal range, hence the blood glucose regulation was supposed to be normal. Accordingly, the lack of observed effect on blood glucose regulation due to the CPH was not surprising. Previous trials investigating fish protein hydrolysates on glucose regulation have included overweight and/or obese individuals with higher BMI than those subjects we included,[47,61,62] hence these study populations were more likely to have an abnormal glucose metabolism making the findings more distinct. We chose to include individuals presumed to have a normal blood glucose response in the current study, hence the lack of effect on postprandial blood glucose levels were as expected. The observed effect on the postprandial insulin concentrations in healthy subjects can arguably be relevant for further investigations, preferably in studies lasting over a period of time and in individuals with impaired blood glucose regulation.

As highlighted in the introduction, few studies have reported on the specific effect of a fish protein hydrolysate on glucose metabolism. Hence, our results adds new knowledge to the field of research regarding novel utilisation of marine recourses and residual material from the fishing industry. Of note, several studies investigating the effects of supplementing the diet with low doses of protein hydrolysates from other protein sources than fish (such as the milk protein casein) have reported promising results of protein hydrolysate supplementation both in patients with T2D[182-184] and gestational diabetes.[185] Ingestion of a supplement with hydrolysed casein together with a meal rich in carbohydrates has been reported to reduce postprandial glucose concentrations and improve glycaemic control in several trials with T2D patients.[182-184]

In addition, supplementation with a drink containing 8.5 g casein protein hydrolysate twice a day for 8 days in 26 women with gestational diabetes, did moderately reduce plasma glucose levels postprandially when compared to placebo.[185] These findings highlight the interest of further investigations of a fish protein hydrolysate supplement in target groups with impaired glucose metabolism. Based on the several disadvantages of pharmacological treatment of diabetes, such as risk of weight gain and hypoglycaemia, alternative approaches to achieve glycaemic control is of great interest.[186]

6.1.1 Interpretation of the reduced postprandial insulin concentration

Of note, it has to be highlighted that the differences between the postprandial insulin concentrations after CPH and after control in the current study are quite moderate, and the clinical implications of our findings can be discussed. It is likely that the statistically significant difference observed ($p=0.032$) is not clinically relevant, however it implies an effect of interest for further investigation targeting a study population with an abnormal glucose regulation, such as patients with metabolic syndrome or T2D. Based on previous findings on glucose regulation related to cod protein intake in animal studies,[27,187] we hypothesize that the CPH might increase the insulin-stimulated glucose uptake in the muscle by enhance mechanisms involved in the glucose transporter system, such as increase translocation of GLUT4 to the cell surface. This hypothesis requires further investigations in a target group before any conclusions can be drawn.

6.2 Effects on outcomes related to appetite (Paper II)

Paper II is based on the secondary outcomes in Study 1; Evaluation of the effect of CPH on outcomes related to appetite regulation. We observed no effect of the CPH for any of the outcomes related to appetite, neither postprandial concentrations of acylated ghrelin or self-reported sensation related to hunger, satiety and feeling of fullness. Accordingly, we were unable to confirm our hypothesis that one low dose of CPH would suppress the ghrelin concentrations postprandially and contribute to a reduced feeling of hunger.

Based on our findings in Paper I, were a reduced postprandial insulin concentration after one supplementation with CPH before a meal was observed, we aimed to investigate whether the alterations in insulin concentrations could be linked to, or be caused by, altered ghrelin concentrations. As we discuss in Paper II, the major effects of ghrelin is linked to mechanisms involved in promoting food intake and avoid starvation to prevent hypoglycemia, such as suppress insulin secretion and stimulate growth hormone secretion to restrict peripheral glucose uptake,[188] and it has previously been reported an inverse correlation between postprandial insulin and plasma ghrelin concentrations.[189] Hence, we found it relevant to investigate whether the observed alterations in insulin concentrations could be partly linked to, or explained by, alterations in concentrations of ghrelin.

6.2.1 Dietary protein and satiety

The effects of protein intake in relation to appetite is a well investigated topic, based on the substantial interest from both the industry and consumers to identify specific substances, foods and/or diets that can generate increased satiety and improve weight management.[190] It is acknowledged in the literature that high-protein diets can be used as successful strategies to prevent and/or treat overweight and obesity, through modulations in appetite and the feeling of satiety.[191]

Replacing fats and carbohydrates with proteins in the diet, is suggested to have beneficial effect on satiety. High-protein ad-libitum diets have led to reduced energy intake due to increased satiety, causing unintentional weight loss, when investigated in several clinical trials.[192,193] A meta-analysis reporting on the effect of a high-protein meal on postprandial ghrelin levels concluded that the consumption of a meal high in proteins increased satiety compared to meals high in either fat or carbohydrates.[194] In addition, proteins from fish have been reported to increase satiety in a greater extent than other dietary protein sources when investigated in postprandial studies.[195,196]

Only one small pilot study in fifteen overweight women has previously reported on the specific hunger-regulating effect of a fish protein hydrolysate.[64] Zair et al. found daily supplementation with 2 g protein hydrolysate from blue whiting for fourteen days to significantly reduce sweet-cravings, in addition to glucose levels postprandially.[64] Of note, this study is comparable to our intervention, as they investigated the effect of a low dose of a protein hydrolysate from lean fish. Thus, the effect cannot be attributed a change in nutrient intake, as seen in dietary interventions where more protein are added to the diet, substantially leading to a reduced intake of fats and carbohydrates and hence a change in the dietary composition of nutrient intake. When supplementing the diet with additional protein in form of a hydrolysate in a low dose, one reduce the risk of a potential effect to occure simply due to a change in the composition and nutrient distribution of the diet. The effects reported by Zair et al. suggest that we might would have observed an effect of the CPH if it was investigated in a higher dose and supplemented over a period of time.

6.2.2 Limitations to the study design

As the outcomes related to appetite were secondary outcomes, several aspects of the study design could explain the lack of findings. One could arguably have improved the design by include a more standardized and thorough evaluation of self-experienced hunger as measured by the questionnaires. In addition, inclusion of an ad libitum meal for measurements of actual food intake after CPH and control, served four hours after

the breakfast meal, could have strengthen the design and made it possible to measure the actual caloric intake after CPH versus control. In addition, it would have been of interest to include measurements of other hunger regulating GI hormones, such as ISL5 and GIP, as these hormones also affect appetite and can be related to the insulin concentrations postprandially.

6.2.3 Interpretation of acylated ghrelin concentrations

In addition to the limitations to the design already presented in Paper II, it has to be highlighted that our measurements of acylated ghrelin might hold a methodological weakness. Plasma for the analysis of acylated ghrelin was obtained by centrifugation of full blood, sampled in 4 ml EDTA-K3/Aprotinin blood collecting tubes. As acylated ghrelin is quite rapidly degenerated in plasma, it has been suggested that the best method for quantifying clinical plasma acylated ghrelin levels is to pretreat plasma samples with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF).[197] The guidelines from the manufacturer of the equipment used for samples and analysis of acylated ghrelin in the current study did not include recommendations on addition of AEBSF, thus we did not include pretreatment of AEBSF to the plasma samples in our protocol. Hence, it is possible that the levels we detected in our samples were lower than reality due to degeneration prior to analysis. This should be taken into consideration when interpreting the result. Data for acylated ghrelin values measured at baseline and postprandially after the intake of CPH/control drink and the breakfast meal is shown in **Table 4**.

In human individuals, the circulating concentrations of ghrelin are presumed to be high right before a meal, and then drop to the lowest within 1 hour after food intake before a new rise. This fluctuation is thought to result in a two- to threefold variation in circulating levels of ghrelin in plasma.[198] In our data, we did not include a measure 60 minutes postprandially, hence it is possible that we have missed the lowest level. In addition, the levels of acylated ghrelin varied greatly between individuals, leading to large standard deviations, and we did not observe a two- to threefold rise or decrease in

concentrations. Of note, the concentrations of acylated ghrelin was numerically highest 180 minutes postprandially, and not at baseline after the overnight fasting.

Table 4. Plasma acylated ghrelin concentration of the 41 participants included in the study evaluating the effect of CPH on postprandial acylated ghrelin concentrations after intake of one dose of CPH and control (casein) drink.

Acylated ghrelin (pg/ml)	CPH		Control	
	Mean	SD	Mean	SD
Baseline	97.4	196.3	90.0	194.0
0 min	91.1	202.5	89.7	193.0
20 min	84.3	199.6	84.7	198.4
40 min	86.1	204.8	84.2	199.7
80 min	83.4	189.3	86.2	201.7
180 min	101.4	199.5	95.4	192.7

CPH, cod protein hydrolysate; SD, standard deviation

It has been suggested that ghrelin levels may change according to pathologic states related to body weight. Several studies have suggested that the fasting levels of ghrelin in plasma are lower in obese than lean individuals,[199,200] and increased levels have been reported in anorexia nervosa and cachexia.[201] Of note, reported ghrelin concentrations seems to vary greatly in existing literature, and no clear reference limits are reported. In addition, the suggested possible actions of which ghrelin affect body weight include not only overproduction or low concentrations, but also increased receptor sensitivity to ghrelin action,[202] a hypothesis making the concentration of less relevance. Wang et al. recently reported that obesity alters the adrenergic and chemosensory signaling pathways that regulate ghrelin secretion in the human gut.[203] They found that obesity altered the sensitivity of the ghrelin secreting cells to glucose in the small intestines, but not in the fundus of the stomach, and that activation of the sweet taste receptors in the gut inhibited the bitter taste signaling of the ghrelin secreting cell. These findings provide new mechanistic insight in the action of the ghrelin producing cell in obese individuals.[203]

Accordingly, today there is a lack of knowledge to clearly interpret the relevance of ghrelin concentrations. We did not observe differences in acylated ghrelin concentrations between genders, a correlation between body weight and the baseline values or differences in postprandial response for those with BMI < 25 compared to those with BMI ≥ 25.

6.3 Effects of different doses of CPH (Paper III)

In Paper III we aimed to investigate the effect of increasing doses of CPH to create a basis for further protocols. Although no statistically significant differences were observed between the four different doses of 10, 20, 30 and 40 mg/kg body weight on postprandial glucose metabolism, our results suggest that the highest dose of 40 mg/kg body weight was more effective than the lower doses. As presented in the introduction, existing literature reporting on the effects of fish protein and fish protein hydrolysate supplements on metabolic outcomes in human individuals have investigated the effects of different set doses of proteins or peptides ranging from 1 to 6 g per day.[61,63,64]

6.3.1 Interpretation of results

To our knowledge, no previous publications have reported on effects of different weight-adjusted doses of fish protein hydrolysates in a dose-range design as presented in the current trial. Hence, the study can be regarded explorative in this manner. Although no clear conclusions can be drawn based on our findings, we suggest that a metabolic effect can be observed in response to a dose corresponding to 3-4 g of fish protein hydrolysate a day. This can be regarded a valuable foundation for further investigations, however there is need for further evaluation of these results before one can suggest a beneficial dose level, if any.

The results presented in Paper III have to be interpreted taking certain limitations in the design into account. Limitations not discussed in detail in the paper are addressed below.

6.3.2 Limitations with the baseline study visit

The study design originally included an additional primary study visit, with baseline measures from a meal without test material (CPH). This visit would be the first of five identical test days including postprandial measures after a breakfast meal. Instead of the test drink with the last dose of CPH from the current week given at the next four study visits, the participants were given a glass of orange juice. A glass of 2 dl of orange juice

was chosen as it contains the same number of calories (100 kcal) and carbohydrate (20 g) as the drinks containing test material. However, due to a mistake in the implementation of the study, the juice serving as control were given together with the breakfast, whereas the test drinks with CPH provided the additional four test days were given 10 minutes before the breakfast meals was served. Accordingly, when analyzing the postprandial measures, the postprandial curve after the baseline/control study visit with orange juice (planned as a control curve) was displaced for both glucose, insulin and GLP-1, when compared to the postprandial curves for these measures after the four different doses. **Figure 6** show the postprandial curves for glucose concentrations, including the control curve not reported in paper III.

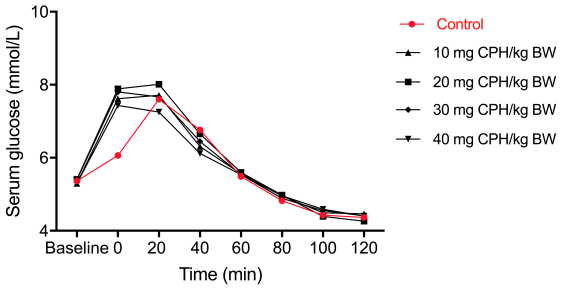


Figure 6. Postprandial glucose concentrations after intake of a glass of orange juice served together with a standardized breakfast meal (control, red curve), and after intake of the test drink with four different doses of CPH given 10 minutes before the standardized breakfast meal (n=31). BW: body weight.

We presume this shift to the right occurred due to the 10 minutes difference in serving of juice (control) and the four test drinks. This could obviously have been avoided by serving the control juice 10 minutes before the breakfast, however this mistake was observed after the study had ended. As this mistake made the control curve non-comparable with the curves after the four doses, we chose to remove the control visit from the results reported in Paper III. Hence, the four different doses is compared to each other, and not to a control. The lack of a control measurement in Paper III can arguably be regarded a limitation, as a placebo or control measure would have strengthened the results.

6.4 Effects on gut health in IBS (Paper IV)

In Paper IV we aimed to investigate whether a supplement with a low dose of CPH could affect gut health in patients with IBS. We observed no effect of the CPH supplementation compared to placebo, when evaluating the outcomes related to symptom severity, gut integrity markers, inflammation and fecal fermentation.

Based on the hypothesis that the CPH contains bioactive peptides capable of induce a local effect in the GI tract, we found it interesting to evaluate the possible effect on gut health in patients with IBS. As it has previously been reported that a diet with lean seafood (including cod) beneficially modulates the gut microbiome composition when compared to a non-seafood diet,[153] we postulated that the CPH supplement could improve symptoms in IBS by influence the gut microbiota composition and function, leading to altered concentrations of fecal metabolites such as SCFAs. In addition, we postulated that supplementation with CPH could reduce the gut permeability and degree of inflammation, measured by altered levels of gut integrity markers and pro-inflammatory cytokines. Our findings do not support these assumptions. According to our knowledge, this is the first trial reporting on the effect of a fish peptide supplement in an IBS population. Despite the lack of distinct results, our findings can be regarded important as they contribute to increased knowledge on this field of research.

6.4.1 Symptom severity and the placebo effect in IBS

According to our results regarding the severity of IBS symptoms as measured by IBS-SSS and scores for quality of life, we observed a reduction of symptoms in both the CPH and the placebo group, with a significant reduction of symptoms after the intervention only in the placebo group. Interventions in IBS patients are known to be influenced by a strong placebo effect.[204] Meta-analyses estimating the placebo response rate in RCTs investigating the effects of pharmacological therapy, dietary interventions, psychological interventions and alternative medicine in patients with IBS all suggests a placebo response rate of around 40% according to symptomatic outcomes.[205-208] This

highlight the importance of interpreting symptom-based results in IBS patients with caution. As no effects of the supplement was observed according to the secondary outcomes (gut integrity markers, inflammatory markers and SCFAs), we assume that the observed reduction in symptom scores in both the CPH group and the placebo group most likely can be attributed a placebo effect, caused by a biased expectation of experiencing symptom relief when participating in a clinical trial and taking a provided supplement.

6.4.2 Amino acids and gut health

There is growing evidence suggesting that dietary proteins can modulate the composition and metabolic activity of the gut microbiota, and that the amino acid composition and digestibility of the proteins in the diet is of great importance for the outcome.[151] The GI tract is the organ in the body with the highest levels of immune activity, and alterations in gut microbiota composition and gut barrier function leading to destruction of the intestinal homeostasis is closely related to the development of several diseases, including GI disorders such as IBD and colorectal cancer.[209,210] Increasing evidence from both animal and human trials highlight the role of amino acids as key regulators of intestinal health, capable of regulating the mucosal barrier function and integrity of tight junctions in the intestines, as well as influence anti-oxidative responses and the gut microbiota composition and activity.[156,211]

As presented in **Table 3** and discussed in paragraph 5.1.2, the CPH supplement investigated in this PhD-project contains a relative high concentration of the essential BCAAs leucine, isoleucine and valine. It is suggested in the literature that optimizing the dietary BCAA levels can have a beneficial effect on parameters related to several health outcomes, including intestinal health.[156] BCAA-supplementation in piglets resulted in improved gut barrier function and intestinal immune defense, suggesting that BCAAs might serve as a regulator capable of promote intestinal development and improve gut health,[212] whereas BCAA-supplementation in rats caused structural changes in the gut microbiota considered to be beneficial for health.[213] Whether BCAAs

provided in the form of a hydrolyzed protein are capable of affecting pathways in GI tract of importance for gut health when supplemented to human subjects in low doses, has to our knowledge previously not been investigated.

A few studies have highlighted and suggested a role of amino acid deprivation in IBS pathogenesis, focusing on the essential amino acids' glutamate and tryptophan, in particular. Depletion of these are shown to lead to atrophy of intestinal epithelial cells and increased intestinal permeability,[214] as well as reduced serotonin production and suggestively disruption in the tryptophan-kynurenine balance causing altered interaction between oxidants and antioxidants,[215] respectively. Although the CPH investigated in this project has a low concentration of both glutamate and tryptophan, the suggested mechanisms pose interesting ideas for further investigations of effects of different amino acids in IBS pathogenesis, as well as effects on gut health in general.

6.4.3 Markers in IBS

As discussed in Paper IV, the significant inverse correlation observed between the total baseline symptom scores and SCFA concentrations should be interpreted with caution, as this connection cannot be concluded on based on previous research, and the literature pose conflicting results.[130,149] Although the relevance of SCFAs as a marker for IBS is still unclear, the existing literature suggests that the SCFA pattern is different for the different IBS-subtypes.[149,178] A study comparing IBS patients to healthy controls reported that the total concentration of SCFAs did not differ between the groups, however when looking into the different subtypes, the total SCFA level was significantly lower in IBS-C patients than those with IBS-D and IBS-M.[178]

There is a lack of literature reporting on "normal" levels of the implemented gut integrity markers in an IBS population, and the use of these as biomarkers are discussed.[142,146] Interestingly, novel results from Linsalata et al. suggest that alterations in the small-intestinal permeability might contribute to the pathogenesis of diarrhea predominant IBS.[137] When investigating the levels of several gut integrity markers in a case-control

study comparing IBS-D patients with healthy controls, they managed to identify two distinct subtypes of IBS-D according to degree of intestinal permeability, as measured by increased or normal ratio of lactulose/mannitol in urine. They reported that the IBS-D subtype with increased small-intestinal permeability had significantly higher levels of iFABP, LPS and IL-6 than those IBS-D patients with normal intestinal permeability.[137] Of note, other markers than the ones included in our study could have been of interest when investigating gut integrity, such as the tight junction proteins occludin and claudin-1, in addition to excretion of different carbohydrate/sugar ratios in urine.[216]

Despite the lack of observed effects in response to supplementation with CPH in the IBS population, our findings in Paper IV contribute with new knowledge and data regarding fecal fermentation and gut integrity markers in an IBS population. Although more research is needed before the value of these markers IBS can be concluded on, this study can be used as a basis for designing future studies investigating the effect of other potentially beneficial peptide supplements on gut health in IBS patients.

7. Conclusion

- Preprandial supplementation with 20 mg/kg body weight CPH reduced the postprandial insulin concentration in healthy, middle-aged to older adults without affecting postprandial glucose concentration, GLP-1 levels, concentration of acylated ghrelin or self-reported sensations associated with appetite when compared to control.

- When investigating the effect of four different doses of CPH given to older adults for seven days, no significant differences were observed between the doses corresponding to 10, 20, 30 and 40 mg/kg body weight CPH per day. However, the tendency was that the highest dose of 40 mg/kg body weight to a greater extent decreased postprandial glucose and insulin concentrations than the lower doses.

- When 2.5 g CPH was supplemented to IBS patients for six weeks, no significant effects were observed in response to the CPH intervention when compared to placebo, either for symptom severity, gut integrity markers, inflammatory markers or fecal fermentation measured by concentrations of SCFAs.

These findings only partly confirm our hypothesis suggesting that CPH would beneficially affect glucose metabolism and appetite regulation in healthy, middle-aged to older adults, that it would be a dose-response relationship between different doses of CPH supplementation and changes in the glucose metabolism, and that supplementation with CPH would beneficially affect gut health in patients with IBS.

In summary, we observed beneficial alterations in insulin concentrations after supplementation with CPH and a dose of 40 mg/kg body weight is likely to be more effective than lower doses, but supplementation with CPH was not found to influence outcomes related to hunger regulation in healthy subjects or gut health in patients with IBS in the doses investigated in the included studies.

8. Future perspectives

The three clinical trials included in the current PhD-thesis were designed to investigate the health effect of a protein hydrolysate from cod, focusing on outcomes related to glucose metabolism and appetite in healthy individuals and gut health in patients with IBS. Our results pose novel opportunities for further research:

- As an optimal supplementation dose with a hydrolysate from lean fish cannot be concluded on based on our findings, future trials should arguably investigate the effect of higher doses than the ones included in the studies presented here.

- According to our findings reported on insulin concentrations in Paper I, we suggest to further evaluate the effect of a CPH supplement in a target group with impaired glucose metabolism, preferably over a period. Patients with metabolic syndrome and/or diet-controlled T2D would be of particular interest.

- We did not analyze the CPH for the content of specific bioactive peptides. The identification of sequences with known bioactive effects could be of interest if the supplement was to be investigated in future clinical trials.

- If further investigating the effect of a similar supplement or other suggested bioactive peptides on gut health in patients with IBS and/or other GI disorders, one should aim to evaluate the effect of peptides holding sequences with known gut-specific and/or anti-inflammatory properties.

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RESEARCH ARTICLE

Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: a double-blind cross-over trial

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Abstract

The increased prevalence of lifestyle diseases, such as the metabolic syndrome and type 2 diabetes mellitus (T2DM), calls for more knowledge on dietary treatments targeting the specific metabolic pathways involved in these conditions. Several studies have shown a protein preload before a meal to be effective in lowering the postprandial glycaemic response in healthy individuals and patients with T2DM. The aim of the present study was to assess the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism in healthy, middle-aged to elderly subjects. This double-blind cross-over trial (*n* 41) included two study days with 4–7 d wash-out in between. The intervention consisted of 20 mg of MPH (or casein as control) per kg body weight given before a breakfast meal. The primary outcome was postprandial response in glucose metabolism, measured by samples of serum glucose, insulin and plasma glucagon-like peptide 1 (GLP-1) in 20 min intervals for 180 min. In a mixed-model regression analysis, no differences were observed between MPH and control for postprandial glucose concentration (mean difference: −0·04 (95 % CI −0·17, 0·09) mmol/l; $P = 0·573$) or GLP-1 concentration (mean difference between geometric means: 1·02 (95 % CI 0·99, 1·06) pmol/l; $P = 0·250$). The postprandial insulin concentration was significantly lower after MPH compared with control (mean difference between geometric means: 1·067 (95 % CI 1·01, 1·13) mIU/l; $P = 0·032$). Our findings demonstrate that a single dose of MPH before a breakfast meal reduces postprandial insulin secretion, without affecting blood glucose response or GLP-1 levels, in healthy individuals. Further studies with repeated dosing and in target groups with abnormal glucose control are warranted.

Key words: **Marine protein hydrolysate: Fish protein: Marine peptides: Glucose metabolism**

The proportion of the population with health problems related to overweight and obesity is constantly increasing worldwide, and this constitutes a great risk factor for several lifestyle diseases such as insulin resistance, the metabolic syndrome and type 2 diabetes mellitus (T2DM)[(1)]. The ability of the body to control postprandial glucose metabolism is decisive

Abbreviations: BCAA, branched-chain amino acids; GLP-1, glucagon-like peptide 1; MPH, marine protein hydrolysate; T2DM, type 2 diabetes mellitus.

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for health. Several dietary treatments for the prevention of postprandial hyperglycaemia in both diabetic and non-diabetic individuals have been suggested, but the necessary lifestyle and diet changes are challenging, and continue to lack adherence[2]. There is a need for more knowledge on dietary treatments targeting the specific metabolic pathways involved in overweight, obesity, the metabolic syndrome and T2DM.

Diets relatively high in protein (18–30 % energy) have been shown to be effective in the management of obesity due to suppression of appetite[3], and are further suggested to reduce postprandial blood glucose in both healthy individuals and patients with impaired glucose metabolism[4]. Several trend diets have over the last decades included high-protein diets to reduce weight and suppress insulin response[5,6], but the long-term effects of high-protein diets are unknown[7,8]. Furthermore, several studies have shown a protein preload before a meal to be effective in lowering the postprandial glycaemic response both in T2DM patients and healthy subjects[9–13].

Due to limited access to high-quality protein in the world, it is neither sustainable nor possible for the world's population to increase the proportion of protein in the diet. Thus, the potential benefit of altering the source and quality of protein, rather than increasing the amount, is of great interest. Marine resources in excess should be evaluated as a possible high-quality protein source for human consumption[14].

Previous studies in rats and human subjects have shown that the intake of both fish proteins and marine protein hydrolysates (MPH), even in low doses, has a desirable effect on insulin sensitivity and postprandial glucose[7,15–19], lipids in serum and adipose tissue, bile acids, fatty acid composition and growth, and possibly has antihypertensive and immune-modulating effects[14,19–23]. It is indicated that MPH may contain marine bioactive compounds with potentially important biological effects in humans, beyond the known effect of protein as a source of amino acids[24,25]. The use of MPH as a dietary supplement with similar or better health benefits than a regular fish meal could be both cost-effective, environmentally friendly and sustainable. A low dose of MPH is presumed to be effective due to the content of bioactive peptides not equally present in other protein sources.

Thus, the present study was designed to assess the effect of a single, low dose of MPH before a meal on postprandial glucose metabolism in healthy, middle-aged to elderly subjects.

Subjects and methods

Trial design

The study was a double-blind cross-over trial, including two different study days, with a 4–7 d wash-out period in between. The intervention implemented 20 mg of MPH per kg body weight (test material) or control (casein). MPH or casein powder (identical, both flavoured with lemon) was mixed with water and taken before a standardised breakfast meal, in randomised order. The primary outcome was postprandial response in glucose metabolism, measured by venous samples of serum glucose and insulin, and plasma glucagon-like peptide 1 (GLP-1). The secondary outcome was adverse events measured by symptom questionnaires.

This 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 Committees for Medical and Health Research Ethics of Central Norway (2017/1794). Written informed consent was obtained from all subjects. The trial was registered at clinicaltrials.gov as NCT03669796.

Participants

Participants were recruited through advertisements on the Internet and posters at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018. Potential participants were interviewed for general eligibility and compliance with inclusion and exclusion criteria, and suitable candidates were invited for a further screening visit. A total of forty-one healthy and active individuals between 41 and 64 years old were included in the study (male, *n* 15; female, *n* 26). The inclusion process is depicted in Fig. 1.

Inclusion criteria were aged 40–65 years old and BMI 20–30 kg/m^2. Exclusion criteria were fish allergy, pharmacologically treated diabetes mellitus, elevated blood pressure, chronic diseases (that might affect the evaluation of the study endpoints) and acute infections. The participants were instructed not to take any nutritional supplements containing *n*-3 fatty acids for 1 week before the study start, and while participating in the study.

Study protocol

The participants came to the research units on two different occasions, with a 4–7 d wash-out period (Fig. 2). A clinical examination by a physician, baseline biochemistry and measures of height, weight and blood pressure were done before inclusion. The level of physical activity was assessed, and participants were instructed not to change the level of physical activity or diet composition during the study period.

A 3-d and 1-d prospective dietary record was filled out prior to study days 1 and 2, respectively. On the day preceding each study day, the participants were provided with a standardised evening meal (oatmeal, rice or barley porridge) instructed to be eaten before 20.30 hours, followed by fasting until the next morning.

On study days, the participants came to the research units between 08.00 and 09.00 hours. After blood samples, they were served a drink with MPH or control, before a breakfast meal was given. The first post-meal sample (0 min sample) was taken 15 min after the breakfast was served.

The standardised breakfast meal consisted of two slices of semi-coarse bread (50 % whole wheat, 80 g bread), 10 g margarine, 20 g strawberry jam and 20 g white cheese, providing a total of 355 kcal (1485 kJ), 41 g carbohydrate, 12·5 g protein and 15 g fat. The drink provided on average 35·9 g carbohydrate and 145 kcal (607 kJ). Thus, including the drink, the breakfast provided in total 500 kcal (2092 kJ) and 77 g carbohydrate. The amount of energy and carbohydrates in the

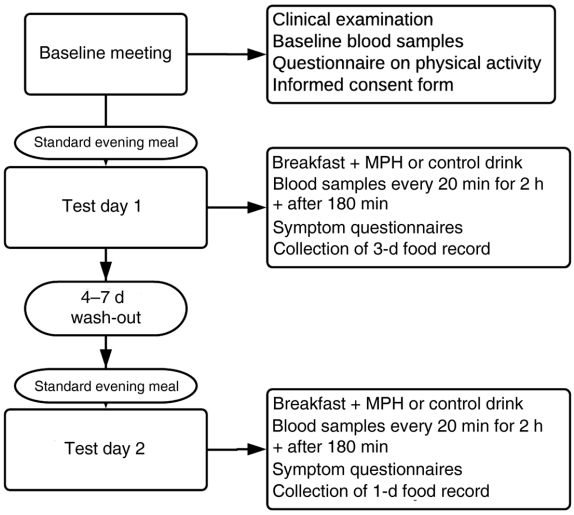

Fig. 1. Flowchart depicting the inclusion process for the study evaluating the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism in healthy individuals aged 40–65 years. Participants were recruited through advertisements on the Internet and posters at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018.

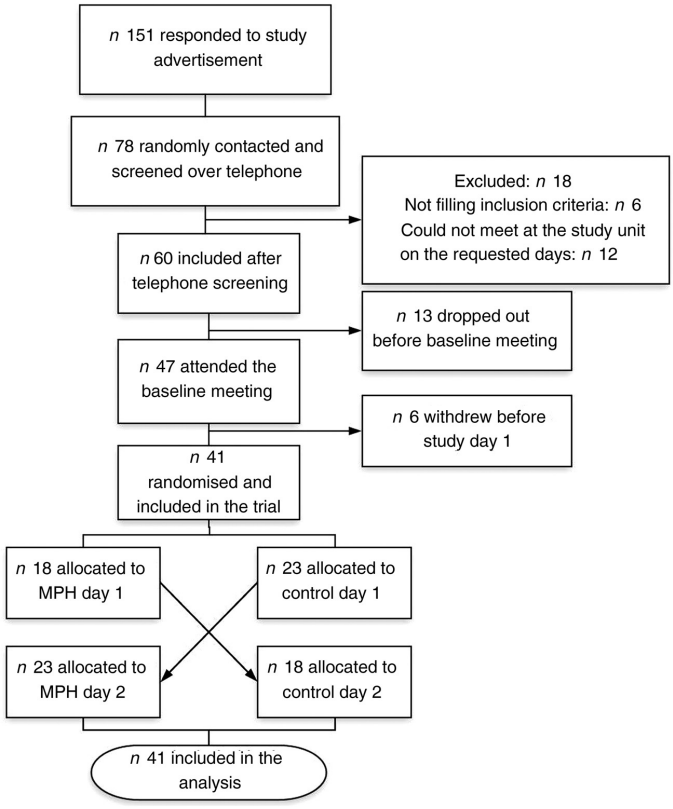

Fig. 2. Study protocol for the evaluation of the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism. We included forty-one healthy subjects (age range 40–64 years).

breakfast was calculated to induce an adequate blood glucose response. No coffee or tea was served, but water *ad libitum*.

The participants spent 4 h at the research units to allow for repeated sampling of blood, at 20 min intervals until 180 min, and monitoring of blood pressure.

Assessments

Assessment of the participants' medical history, and measurement of biochemical variables and safety parameters were conducted at baseline.

During the study days, serum glucose and serum insulin were measured at baseline and every 20 min for 2 h (120 min), with a final sample at 180 min. GLP-1 was measured at baseline, time 0, 20, 40, 80 and 180 min. Blood pressure was measured at baseline, after 40 min and after 180 min, as a safety parameter.

Two questionnaires evaluating the participants' self-experienced symptoms were implemented to identify possible adverse events during each study day. A visual analogue scale was filled out six times during the study day, and a questionnaire validated for the evaluation of gastrointestinal symptoms (Kane) was filled out at baseline and at the end of each study day[26].

Estimation of nutritional intake

Calculations of energy and macronutrient intake were performed using *Kostholdsplanleggeren* (Norwegian Food Safety Authority and The Norwegian Directorate of Health, Oslo, Norway)[27]. The dietary records were used to evaluate the composition of the baseline diet, to map the participants' regular meal pattern and to compare the days prior to each study day according to energy intake.

Test materials

The MPH and casein powder were delivered from the manufacturer (Firmenich Bjørge Biomarin AS) in neutral bottles coded with participant number and study day. The bottles were coded by a person not involved in the implementation of the study and randomised according to a randomisation list. Both study participants and all persons involved in study conduction and analysis were blinded. The powder contained 4 % protein (MPH raw material or casein) and 96 % carbohydrate (maltodextrin). It was flavoured with lemon, but otherwise neutral. It was not possible to identify the active ingredient from the control, according to flavour or appearance. Each participant was given 20 mg/kg body weight of MPH or control. The drinks were made isonitrogenous, and equal amounts of N in the form of casein were added to the control drink. This was done to avoid any bias due to difference in N content between the MPH drink and the control drink. The amount of protein (N x 6·25) in both drinks was on average 1·6 g, constituting only a small fraction of the total protein content of the standardised breakfast meal. Casein was chosen as the control as it has previously shown to not affect blood glucose or insulin sensitivity when compared with proteins from cod and soya[28].

Table 1. Molecular weight distribution in the dry and solubilised marine protein hydrolysate produced from meat of Atlantic cod (*Gadus morhua*)

Molecular weight (Da)	Amino acid moieties	g/100 g soluble peptides	g/100 g in the spray-dried powder
>10 000	–	<0·1	<0·1
10 000–8000	88–71	0·1	0·1
8000–6000	70–53	0·6	0·5
6000–4000	52–36	2·1	1·9
4000–2000	35–18	7·2	6·3
2000–1000	17–10	14·8	13·0
1000–500	9–5	21·0	18·5
500–200	4–2	27·0	23·8
<200	≤2	27·2	24·0

The MPH powder was made by Firmenich Bjørge Biomarin AS by hydrolysing fish meat of Atlantic cod (*Gadus morhua*) with Protamex® (Novozymes AS) followed by spray drying of the soluble part of the enzyme digest. The MPH raw material contained approximately 89 % protein by weight, <0·2 % fat, 0 % carbohydrate, <3·0 % water, 10 % ash, 0·1 % NaCl, 1·7 % Na and 0·07 % chloride. Free amino acids accounted for 4·77 % of total amino acids in the MPH, and the essential amino acids:non-essential amino acids ratio was 0·70. Analysis of the molecular weight distribution (Table 1) shows that about 90 % of the peptides in the fish protein hydrolysate have a molecular weight of 2000 Da or less (eighteen amino acids or fewer), about 75 % of 1000 Da or less (ten amino acids or fewer) while about 55 % have a molecular weight of 500 Da or less (five amino acids or fewer). Approximately 25 to 30 % of the peptides have a molecular weight less than 200 Da, which represents small dipeptides and free amino acids.

The casein contained approximately 88 % protein. The amino acid composition of MPH and casein used as control is presented in Table 2 (data obtained from Firmenich Bjørge Biomarin AS).

The MPH was analysed at the Allergy Laboratory (Haukeland University Hospital, Bergen, Norway) for allergenicity of the hydrolysate. Direct ELISA showed insignificant reactivity of specific IgG and IgE to the hydrolysate in comparison with the reactivity against cod allergen. The allergenicity of the hydrolysate was so low that it was considered unsignificant.

Analysis of blood samples

Baseline biochemistry was analysed according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital (Bergen, Norway) and the Department of Medical Biochemistry, Ålesund Hospital (Ålesund, Norway).

Glucose and insulin were measured in serum according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital (Bergen, Norway). Serum was obtained by centrifugation of full

Table 2. Amino acid and taurine composition of the marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) and the casein control used in the present study

	Total amino acids (mg/g)	
Amino acid	MPH	Casein (control)
Alanine	47·8	28·9
Arginine	51·1	32·0
Aspartic acid	73·3	70·8
Asparagine	0·38	N/A
Glutamic acid	125·0	213·5
Glutamine	0·78	N/A
Glycine	50·9	15·9
Histidine	13·5	24·4
Hydroxyproline	1·0	N/A
Isoleucine*	30·1	46·1
Leucine*	60·3	86·1
Lysine*	71·3	76·3
Methionine	22·1	25·8
Phenylalanine	23·2	47·2
Proline	29·7	95·3
Serine	36·0	50·3
Taurine	6·6	N/A
Threonine	30·9	38·8
Tryptophan	6·0	11·0
Tyrosine	22·7	47·8
Valine	36·9	59·4

N/A, not available.
* Branched-chained amino acids.

blood at 2000 ***g*** at room temperature (20°C) for 10 min after 30–60 min of coagulation, using serum separator cloth activator tubes. Samples were aliquoted and stored frozen at −80°C prior to analyses.

Plasma for GLP-1 determination was obtained by centrifugation of EDTA full blood at 1800 ***g*** at −4°C for 10 min within 20 min after blood sampling. To EDTA blood sampling tubes were added 10 µl dipeptidyl peptidase-4 inhibitor (DPP4-010; DRG Diagnostics) per ml EDTA blood prior to sampling. GLP-1 plasma was aliquoted and stored frozen at −80°C prior to analysis. The GLP-1 analyses were performed using an ELISA kit from IBL International GmbH (GLP-1 (7–36) active ELISA, reference RE53121).

Statistical analysis

Statistical analysis was performed using SPSS software (IBM SPSS Statistics 24) and GraphPad Prism version 7.0 (GraphPad Software, Inc.). The Shapiro–Wilk test was used to assess normal distribution. Mixed-model regression analysis was conducted to evaluate the difference between MPH and control. Non-normally distributed data were log-transformed before analysis (insulin and GLP-1) and are presented as log mean and back-transformed values. Paired *t* tests were used to evaluate differences in nutrient intake between study days. Two-way ANOVA with repeated measures was used to evaluate differences between each time point. Graphical work was conducted in GraphPad Prism. *P* values <0·05 were considered statistically significant.

The sample size was not calculated according to a power analysis, due to lack of similar studies. Previous research reporting on the effect of cod proteins in human subjects is based on whole fish[16] or long-term use of fish protein supplement[17,29]; thus we did not find any data adequate for making a basis for a power analysis representative for our design. We decided to include forty participants (forty-one were included), a number higher or similar to previously reported in studies on cod protein[16,17,29].

Results

Participant characteristics

Overall, forty-one participants completed the trial, of whom twenty-six were female. Mean age was 51 (SD 6) years, range 40–64 years. Mean BMI was 25·2 (SD 3) kg/m^2. The recorded mean energy intake (2084 (SD 504) kcal/d; 8719 (SD 2109) kJ/d) was lower than the estimated energy need (2605 (SD 392) kcal/d; 10899 (SD 1640) kJ/d) at baseline. The standardised breakfast provided on the study days (500 kcal (2092 kJ)) covered 19·6 (SD 2·9) % of the participants' total energy need. All baseline biochemistry was within the current reference values. Baseline characteristics are presented in Table 3.

Energy intake

Mean energy intake before study day 1 was 2030 (SD 550) kcal/d (8494 (SD 2301) kJ/d). Mean intake before study day 2 was 2110 (SD 534) kcal (8828 (SD 2234) kJ/d). The energy intake did not differ before the two study days ($P = 0·201$).

Postprandial measurements

Data at each time point are presented in Table 4. In a multivariable, repeated-measures linear mixed-effects regression analysis, no differences were observed between MPH and control for glucose concentration (mean difference: −0·04 (95 % CI −0·17, 0·09) mmol/l; $P = 0·573$). Mean fasting glucose levels were numerically equal on both study days (5·1 (SD 0·4) mmol/l; $P > 0·999$). The peak in glucose concentration

Table 3. Baseline characteristics of the forty-one participants included in the study at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018*
(Mean values and standard deviations)

Characteristics	Mean	SD
Age (years)	51·0	6·0
BMI (kg/m^2)	25·2	3·0
Systolic blood pressure (mmHg)	125	18
Diastolic blood pressure (mmHg)	78	11
HbA1c (%)	5·2	0·3
Estimated energy need		
kcal/d	2605	392
kJ/d	10899	1640
Energy intake at baseline		
kcal/d	2084	504
kJ/d	8719	2109
Carbohydrates (g/d)	226·7	68·5
Fat (g/d)	90·2	33·0
Protein (g/d)	92·9	23·6

* Nutritional values are based on mean values from 3-d dietary records.

Table 4. Descriptive statistics* of the forty-one participants included in a study at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018, evaluating the effect of marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism measured by serum glucose, insulin and glucagon-like peptide 1 (GLP-1) during exposure to MPH and control (casein) drinks (Mean values and standard deviations)

		MPH					Control				
Outcome	Time	Mean	SD	Log mean	SD	GM	Mean	SD	Log mean	SD	GM
Glucose (mmol/l)	Baseline	5·1	0·4				5·1	0·4			
	0 min	6·5	0·9				6·7	0·8			
	20 min	7·6	1·6				7·4	1·5			
	40 min	6·5	1·8				6·2	1·9			
	60 min	5·4	1·4				5·4	1·6			
	80 min	4·9	1·2				5·1	1·4			
	100 min	4·6	1·1				4·7	1·2			
	120 min	4·5	1·1				4·4	1·0			
	180 min	4·4	0·6				4·3	0·6			
Insulin (mIU/l)†	Baseline	6·4	5·8	1·6	0·7	4·9	6·1	5·6	1·5	0·7	4·6
	0 min	33·8	34·3	3·2	0·9	23·8	34·9	30·4	3·3	0·7	27·5
	20 min	69·6	52·7	4·0	0·6	57·1	68·0	47·7	4·0	0·6	56·3
	40 min	64·8	51·1	4·0	0·6	52·0	70·3	53·6	4·0	0·7	55·1
	60 min	57·2	46·2	3·8	0·6	45·7	61·4	49·0	3·9	0·6	49·3
	80 min	42·9	33·9	3·6	0·6	35·0	51·7	47·3	3·7	0·7	39·7
	100 min	36·4	39·3	3·3	0·7	26·9	40·8	42·0	3·4	0·7	31·3
	120 min	28·6	31·3	3·0	0·8	20·6	30·1	37·9	3·0	0·8	21·1
	180 min	12·2	17·7	2·1	0·9	8·0	12·4	15·1	2·2	0·8	8·7
GLP-1 (pmol/l)	Baseline	6·2	9·5	1·5	0·6	4·4	6·2	9·5	1·5	0·6	4·4
	0 min	8·1	9·1	1·9	0·6	6·5	8·8	9·9	1·9	0·6	7·0
	20 min	8·0	9·4	1·9	0·5	6·4	7·9	9·3	1·8	0·6	6·2
	40 min	7·2	9·1	1·7	0·6	5·6	7·3	9·0	1·7	0·6	5·7
	80 min	6·9	10·3	1·6	0·6	5·0	7·0	9·6	1·6	0·6	5·2
	180 min	6·8	10·1	1·6	0·7	4·8	6·5	0·8	1·6	0·6	4·8

GM, geometric mean ($\exp^{(\text{log mean})}$).
* Log mean and GM are presented for non-normally distributed data (insulin and GLP-1). Glucose values are only presented as means and standard deviations due to approximately normal distribution.
† In a mixed-model linear regression analysis, the insulin levels were significantly lower after intake of MPH than control ($P = 0{\cdot}032$).

(C_{max}) occurred 20 min after the meal for both MPH and control and was numerically higher after MPH than after the control drink (7·6 (SD 1·8) *v.* 7·4 (SD 1·5) mmol/l, respectively; $P = 0{\cdot}997$). The AUC was compared for the nine glucose measurements. The AUC for the glucose concentration was numerically equal between MPH (1078 (95 % CI 956·0, 1199·0) mmol/l × min) and control (1068 (95 % CI 944·8, 1190·0) mmol/l × min; $P = 0{\cdot}910$).

The insulin concentration was significantly lower after MPH compared with control (mean difference between geometric means: 1·067 (95 % CI 1·01, 1·13) mIU/l; $P = 0{\cdot}032$). Mean fasting insulin levels were numerically higher before MPH (6·4 (SD 5·8) mIU/l) than control (6·1 (SD 5·6) mIU/l; $P > 0{\cdot}999$), but the insulin concentration peaked at a lower level and at 20 min (69·6 (SD 52·7) mIU/l) after MPH whereas the peak after the control drink was numerically higher and occurred at 40 min (70·3 (SD 53·6) mIU/l). Women had significantly lower insulin concentrations than men (mean difference between geometric means: 0·65 (95 % CI 0·45, 0·93) mIU/l; $P = 0{\cdot}020$), irrespective of intervention.

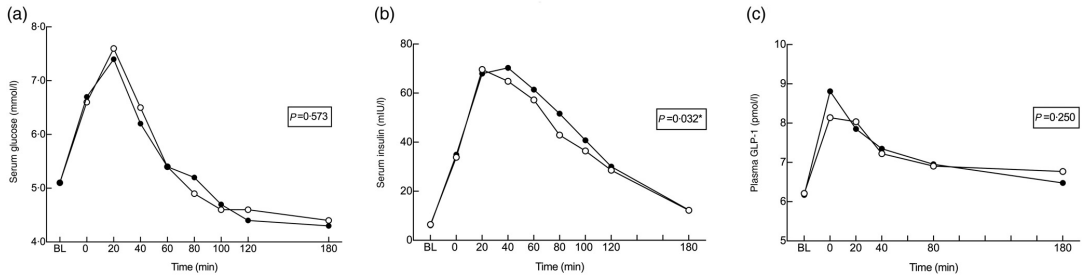

Fig. 3. Metabolic response for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) concentrations after intake of a standardised breakfast meal supplemented with a drink containing either marine protein hydrolysate (MPH; –○–) or control (casein; –●–). Results are presented for forty-one healthy subjects. The study had a cross-over design and all subjects received both treatments in random order. Time point 0 min shows values measured right after the intake of breakfast and test material. Values are means and *P* values are based on a multivariable, repeated-measures linear mixed-effects regression analysis. BL, baseline.

No differences were observed between MPH and control for GLP-1 concentration (mean difference between geometric means: 1·02 (95 % CI 0·99, 1·06) pmol/l; $P = 0·250$). Mean fasting GLP-1 levels were numerically equal on both study days (6·2 (SD 9·4) pmol/l; $P > 0·999$). The peak occurred right after intake of breakfast and test drink (0 min) and was lower after MPH (8·1 (SD 9·1) pmol/l) than after control (8·8 (SD 9·9) pmol/l; $P = 0·092$). Results are presented in Fig. 3.

Adverse events

No adverse events were reported in the questionnaires or otherwise observed.

Discussion

The study was designed to investigate the effect of a low dose of MPH on postprandial glucose metabolism in healthy individuals. Our hypothesis was that supplementation with MPH before a meal would beneficially affect the glucose response, insulin and GLP-1 concentration compared with control. We found that a single dose of 20 mg/kg body weight MPH pre-meal supplement significantly lowered the postprandial insulin response. Although we did not observe a reduction in postprandial blood glucose values and GLP-1 concentrations, we postulate that our findings could indicate a potential beneficial effect of MPH in individuals with reduced insulin sensitivity. We hypothesise that MPH may enhance the insulin sensitivity and affect other mechanisms involved in the blood glucose uptake in peripheral tissue. The study participants were healthy individuals with HbA1c levels within the normal range (Table 2), thus one would expect normal blood glucose concentrations after a meal. We speculate that the effect of MPH on postprandial glucose metabolism will be more distinct if further investigated in individuals with the metabolic syndrome or T2DM.

The target of nutritional diabetic and pre-diabetic treatment is to maintain a blood glucose level within the normal range. Several studies have previously shown different sources of protein preload before a meal to reduce the postprandial glycaemic response, both in healthy and diabetic individuals[9–13]. However, to our knowledge, data on the specific acute effect of a fish protein hydrolysate supplement prior to a meal has previously not been published.

Our finding is consistent with a previous study, showing lower postprandial insulin C-peptide levels after a 7-d intervention with cod[30]. Furthermore, Ouellet *et al.*[16] have previously demonstrated that a diet rich in cod improved insulin sensitivity in nineteen insulin-resistant individuals, when compared with a diet rich in other animal protein sources. Also, it has previously been demonstrated that cod protein-fed rats, in comparison with casein-fed and soya-fed rats, are protected against the development of insulin resistance and hyperglycaemia induced by diets rich in fat and sucrose[28]. This effect was related to enhanced insulin-stimulated glucose uptake in muscle cells, but not in adipose tissue. It is indicated that amino acids derived from cod protein can increase the insulin-stimulated glucose uptake in muscle cells by acting directly on the glucose transport system[28]. Investigations of the mechanisms promoting this positive effect of amino acids from cod revealed that dietary cod protein restored insulin-induced activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway and improved translocation of GLUT4 to the T-tubes in skeletal muscle cells[31]. The glucose transporter protein GLUT4 facilitates the uptake of glucose into the cell when expressed at the cell surface, and it has been proposed that a reduced translocation of GLUT4 to the T-tubules leads to the development of insulin resistance[31]. It is proposed that the amino acids derived from cod protein, in comparison with amino acids derived from other protein sources, facilitate a unique pathway leading to the increased expression of GLUT4 in the T-tubules and enhanced insulin sensitivity[31].

The assumed beneficial effect of the amino acids derived from cod can possibly be linked to the high concentration of branched-chain amino acids (BCAA). It has previously been demonstrated that serum levels of the BCAA leucine, isoleucine and valine, as well as the amino acid lysine, is correlated with the insulin response[32]. The effect has been linked to the increase of hormones such as glucose-dependent insulinotropic polypeptide and GLP-1[33]. Although it is established that the BCAA leucine and isoleucine are the major amino acids affecting blood glucose homeostasis, the effect has not been observed when the amino acid concentration is low[34]. Interestingly, a significant stimulation of glucose uptake in muscle cells by the PI3K/Akt pathway has been observed when the BCAA were administered as dipeptides in low concentrations[35]. The MPH used in our trial have a high concentration of BCAA (Table 1), and analysis of the MPH used in the present study shows that about 10 % of the di- and tripeptide fractions are present as leucine- and isoleucine-containing peptides (data obtained from Firmenich Bjørge Biomarin AS). Even though we did not observe an increase of GLP-1 in relation to the intake of single, low dose of MPH, our findings suggest that low concentrations of MPH may increase insulin sensitivity. The casein used as the control has higher concentrations of BCAA than the MPH, but it differs from MPH regarding the fraction present as di- or tripeptides. The casein used in the study is not a hydrolysate, but present as whole protein, and does not contain either peptides or free amino acids. Thus, we assume that the BCAA-containing peptides present in MPH constitute the unique, bioactive effect even when given in low concentrations. We postulate that this is due to the rapid absorption of intact bioactive leucine- and isoleucine-containing peptides via peptide transporters in the upper jejunum and into the blood. It has been shown that other sources of protein, such as casein and whey, are necessary in much higher doses than MPH to achieve significant alterations in the postprandial blood glucose and insulin response[36,37].

One could argue that the control drink should be a true placebo and only contain glucose (maltodextrin), and no protein. However, then it would be possible that the observed effect could simply be due to differences in energy and N content. To avoid this, the control drink contained casein, a protein shown not to affect blood glucose response and insulin

sensitivity when given in low concentrations[28,38,39], to facilitate an isoenergetic and isonitrogenic placebo material. Both the MPH and casein control drinks contained an equal amount of protein, in total on average 1·6 g. This amount is negligible compared with the total protein content of the breakfast meal provided (12·5 g protein); thus the effect of MPH can be attributed to the content of bioactive peptides and not the protein *per se*. In a clinical study comprising of 120, slightly overweight (BMI between 25 and 30 kg/m^2) male and female subjects, Nobile *et al.*[40] showed that oral doses of 1·4 and 2·8 g MPH from the codfish species blue whiting (*Micromesistius poutassou*) taken daily for 90 d increased the blood concentrations of both cholecystokinin and GLP-1. Further, body weight composition was improved in favour of reduced body fat mass. Daily doses higher than 1·4 g did not give any further effects, demonstrating that MPH may show bioactivity in humans when taken orally in the range of 15–20 mg per kg body weight.

Previous studies have investigated the long-term effect of fish protein intake in overweight, obese and/or diabetic individuals. Improvement in postprandial glucose regulation after intake of 750 g fatty fish/week (for 8 weeks) in overweight/obese adults has been demonstrated, but this effect was not observed after intake of lean fish[15]. Similar findings have been reported in T2DM patients; Zhu *et al.*[41] demonstrated that treatment with a fish protein hydrolysate improved glucose and lipid metabolism, resulting in reduced fasting blood glucose, insulin and HbA1c, compared with placebo. Vikøren *et al.*[17] were the first to investigate the specific effect of a fish protein supplement on postprandial blood glucose. They found that low doses of a fish protein supplement from cod (3 and 6 g) for 4 weeks resulted in lower levels of fasting and postprandial glucose, including lower AUC for glucose when compared with placebo, in thirty-four overweight individuals. Another recent study found that supplementation with cod protein for 8 weeks in forty-two overweight and obese individuals had a beneficial effect on postprandial concentration of serum NEFA, but no effect was observed in postprandial glucose or insulin concentration compared with control[29]. The most obvious difference when comparing these studies with our study design is that they evaluated the long-term effect of fish intake/fish protein supplement in overweight and obese patients, while we were interested in the acute effect of a fish protein hydrolysate after a meal in healthy individuals.

There are elements with our design that may have affected the outcome. Previous studies in human subjects have investigated the effect of fish or fish protein supplementation over a longer period of time. Thus, it will be interesting to investigate a potential effect using different doses given over a period of time. Furthermore, we investigated the effect of MPH in healthy individuals, assumed to have a normal glucose response. Our findings indicate that further research should aim to include individuals with hyperglycaemia or abnormal postprandial glucose control. The participants in this study might have been too healthy to find a meaningful effect. The significant lower insulin concentration observed after intake of MPH could be important in patients with reduced insulin sensitivity, thus should be further investigated in a group of patients with the metabolic syndrome and/or T2DM. It has to be considered that 1 week of wash-out for the use of *n*-3 supplements before inclusion might not have been enough; thus the short wash-out period may be regarded as a limitation to our design.

Most previous studies have been performed in rodents, and few data exist on the specific effect of MPH supplement in human individuals. The effect of a low dose of MPH on the postprandial glycaemic response has previously just been hypothesised, and our study is the first to investigate this possible association. Thus, this double-blinded cross-over trial investigating the effect of MPH supplement in human subjects can be regarded valuable for future studies. We suggest that the potential effect of MPH should be investigated over a longer period, with higher doses in patients with impaired glycaemic response and reduced insulin sensitivity.

In conclusion, our findings demonstrated that a single dose of MPH before a breakfast meal reduced postprandial insulin concentration without affecting blood glucose response or GLP-1 levels when compared with control (casein), in healthy, middle-aged individuals. The mechanism for this effect is unknown, and further studies are warranted in target groups with abnormal glucose metabolism.

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H. F. D., C. J., T. H., I. B., J. G. H., D. A. L. H. and G. A. L. designed the present study. H. F. D. and C. J. conducted the research. I. B. analysed the GLP-1 samples. H. F. D. and C. J. wrote the manuscript. E. L. obtained funding and provided administrative, technical and material support. All authors reviewed the manuscript.

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CORRIGENDUM

Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: a double-blind cross-over trial — CORRIGENDUM

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Original text and correction:

ORIGINAL TEXT (page 3, Subjects and methods)

Fig. 1. Flowchart depicting the inclusion process for the study evaluating the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism in healthy individuals aged 40–65 years. Participants were recruited through advertisements on the Internet and posters at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018.

Fig. 2. Study protocol for the evaluation of the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism. We included forty-one healthy subjects (age range 40–64 years).

CORRECTION

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ORIGINAL TEXT (page 8, Acknowledgements)

E. L. is Professor Emeritus at the University of Bergen, Bergen, Norway and the managing director of Science of Firmenich Bjørge Biomarin AS, Ellingsøy, Ålesund, Norway. The other authors declare no conflict of interest.

CORRECTION

E. L. is Professor Emeritus at the University of Bergen, Bergen, Norway, and former Scientific Advisor of Firmenich Bjørge Biomarin AS, Ellingsøy, Ålesund, Norway, where he holds a royalty agreement. The other authors declare no conflict of interest.

Reference

Dale H, Jensen C, Hausken T, *et al.* (2018) Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: a double-blind cross-over trial. *J Nutr Sci* **7**, E33. doi:10.1017/jns.2018.23

II

food & nutrition research

ORIGINAL ARTICLE

Acute effect of a cod protein hydrolysate on postprandial acylated ghrelin concentration and sensations associated with appetite in healthy subjects: a double-blind crossover trial

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Popular scientific summary

- Ghrelin is an appetite-regulating hormone, with high concentrations before a meal and reduced concentrations after a meal. Compounds with the ability to suppress the action of ghrelin may be valuable for weight regulation.
- Fish protein hydrolysates are suggested to contain bioactive peptides capable of affecting glucose metabolism and body weight.
- In this study, no effect of a supplement with cod protein hydrolysate on postprandial ghrelin concentrations or sensations related to appetite was observed.

Abstract

Background: Fish protein hydrolysates are suggested to contain bioactive sequences capable of affecting metabolic pathways involved in the regulation of glucose metabolism and body weight when consumed in low doses. Modulation of the appetite-regulating hormone ghrelin may explain suppression of insulin secretion and weight loss observed in previous studies with fish protein hydrolysates.
Objective: This study aimed to assess the effect of a single, low dose of cod protein hydrolysate (CPH) before a breakfast meal on postprandial acylated ghrelin concentration and sensations associated with appetite in healthy subjects.
Design: In this explorative trial with a crossover design, 41 healthy individuals (15 males and 26 females, age 51 ± 6 years) completed 2 study days separated by 4–7 days of washout. On both study days, a test drink containing 20 mg CPH or casein (control) per kg body weight was given immediately before a standardized breakfast meal. Acylated ghrelin concentrations were measured before test drink/breakfast (baseline) and at time 0, 20, 40, 80, and 180 min postprandially. Sensations associated with appetite were measured by a Visual Analog Scale (100 mm) at baseline and 0, 20, 40, and 180 min postprandially.
Results: Statistically, no difference was observed between CPH and control for postprandial acylated ghrelin concentrations (mean difference geometric mean: 1.05 pg/mL, 95% confidence interval [CI]: 0.97–1.13, $P = 0.266$), or between the total area under the curve (tAUC) for acylated ghrelin after CPH (tAUC = 17518 pg/mL × min, 95% CI: 0–47941) and control (tAUC = 17272 pg/mL × min, 95% CI: 0–48048, $P = 0.991$). No differences were found between CPH and control for sensation of appetite, according to tAUC of postprandial scores for satiety ($P = 0.794$) and the feeling of fullness ($P = 0.996$).
Conclusion: We did not find an effect of a single dose of CPH on postprandial concentrations of acylated ghrelin or sensations related to feeling of hunger, compared to control. Further studies should aim to evaluate the effect of a supplement with CPH given daily over a period of time.

Keywords: *hunger; overweight; marine peptides; gastric hormones; nutrition supplement*

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Ghrelin is a gastric hormone, capable of stimulating hunger and influence energy homeostasis (1). It is a small peptide consisting of 28 amino acids, secreted from neuroendocrine cells in the submucosal layer of the stomach (2). The circulating ghrelin concentration gradually increases before a meal and decreases with feeding (3). Two forms of ghrelin are present in the circulation, acylated and non-acylated ghrelin, of which the acylated form is the one known to activate the ghrelin receptor (2).

Acylated ghrelin is a natural ligand binding to the growth hormone secretagogue (GHS) receptor, leading to stimulation of the secretion of growth hormone (GH), reduction in insulin secretion and glucose tolerance (4–6). Furthermore, it has been shown that acylated ghrelin holds potent adipogenic and orexigenic effects mediated through the GHS receptor located in the central nervous system (CNS) (4). Acylated ghrelin is known to directly activate pathways in the CNS controlling both parasympathetic and sympathetic nerve activity through GHS receptors (7) and possibly indirectly suppresses insulin secretion via neural signaling (8). These qualities have created the idea that compounds having the ability to suppress the action of ghrelin may be valuable for the prevention or treatment of overweight, obesity, insulin resistance, and abnormal lipid and glucose metabolism (9, 10).

In previous studies in rats and humans, it has been observed that the intake of low doses of peptides from fish is capable of beneficially influencing glucose metabolism (11–14), reducing adipose tissue mass, and improving serum fatty acid composition (15, 16), when compared to placebo or casein. In addition, some recent studies have found fish protein hydrolysates to beneficially influence hormones involved in the regulation of appetite, such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1), as well as influence the subjective feeling of craving sweets (17, 18), when compared to placebo in human subjects. The suggested effective daily dose based on the current literature in human subjects ranges from 1 to 6 g per day (13, 18). Consequently, the hypothesized effect of a fish protein hydrolysate is not due to the consumed protein *per se,* which is negligible compared to the normal recommended total daily dietary intake of a healthy individual (e.g. 65–80 g protein per day with body weight 80 kg) (19). A possible mechanism for suppression of postprandial insulin concentration and weight loss could be modulation of postprandial ghrelin concentrations, and a low dose of fish protein hydrolysate is presumed to be effective due to the content of bioactive peptides with unique amino acid sequences (20). In the current trial, we hypothesize that a potential suppressing effect on appetite and postprandial ghrelin levels can be attributed to a high fraction of di- and tripeptides with branched-chain amino acids in the cod protein hydrolysate (CPH), facilitating rapidly absorption from the gastrointestinal tract and possibly capable of influencing pathways involved in the regulation of appetite.

As metabolism and energy expenditure decrease with age, middle-aged individuals often experience weight gain. Thus, middle-aged individuals might benefit from an intervention targeting appetite and hunger regulation. Data on the specific effect of a dietary supplement with a fish protein hydrolysate on ghrelin concentrations and sensations associated with appetite have, to our knowledge, previously not been published. The present study aimed to assess the effect of a single, low dose of CPH before a breakfast meal on postprandial acylated ghrelin concentration and sensations associated with appetite in healthy, middle-aged to elderly subjects.

Material and methods

Data on subjects and methods have been described in detail in a previous publication (14).

Trial design

The study was a double-blind crossover trial, which included two study visits for each subject, with 4–7 days of washout in between. The intervention included serving of a test drink containing 20 mg of CPH per kg body weight (test material) or control (casein) in randomized order, immediately before a standardized breakfast meal was served. The CPH and casein powder were mixed with cold water and served as a drink. The primary outcome of the intervention (postprandial response in glucose metabolism) is reported in a previous publication (14). Here, we report the secondary outcome: acylated ghrelin concentrations measured for 180 min postprandially and subjective sensations associated with appetite.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Regional Committees for Medical and Health Research Ethics of Central Norway (2017/1794). Written informed consent was obtained from all subjects. The trial was registered at clinicaltrials.gov as NCT03669796.

Participants

Subjects were recruited at Haukeland University Hospital and Ålesund Hospital between October 2017 and February 2018. Potential subjects were interviewed for general eligibility and compliance with inclusion and exclusion criteria by telephone. Candidates were invited for a further screening visit.

Inclusion criteria were as follows: age 40–65 years and body mass index (BMI) 20–30 kg/m^2. Exclusion criteria were fish allergy, pharmacologically treated diabetes mellitus, elevated blood pressure, chronic diseases that might affect the evaluation of the study endpoints, and acute infections.

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Study protocol

The screening visit included a clinical examination by a physician, biochemistry tests for safety purposes (leukocyter, trombocytes, hemoglobin, fasting glucose, long-term blood glucose, C-reactive protein, creatinine, sodium, potassium, kidney function estimate, liver enzymes and muscle enzymes) and compliance with inclusion criteria, measuring of height, weight, and blood pressure, as well as assessment of the level of physical activity. The level of physical activity was assessed by asking the participants two questions regarding moderate physical activity and vigorous activity (self-reported). The participants were instructed not to change the diet composition or the level of physical activity during the study period. On the day preceding each study day, the participants received a standardized porridge evening meal to be eaten before 8:30 pm. After this, the subjects were instructed to fast until the next morning and were only allowed to drink water. On study days, the participants came to the research units in a fasting state between 08:00 am and 09:00 am. After the first blood sample, the subjects were served the test drink, before the breakfast meal was provided. Fifteen minutes after the breakfast was served, the first post-meal sample (0 min sample) was taken.

The standardized breakfast meal consisted of two slices of bread (50% whole wheat, 80 g bread), 10 g margarine, 20 g strawberry jam, and 20 g white cheese. This provided a total of 355 kcal (1,485 kJ, 41 g carbohydrate, 12.5 g protein, 15 g fat). The drink contained on average 35.9 g carbohydrate and 145 kcal (607 kJ). Thus, including the drink, the breakfast provided in total 500 kcal (2,092 kJ) and 77 g carbohydrate, equal at both study days. Water was given *ad libitum*, but no coffee or tea was allowed. The subjects spent 4 h in the research units, and repeated sampling of blood was conducted before serving of the test drink and breakfast, and at time 0, 20, 40, 80, and 180 min postprandially.

Assessments

Assessment of medical history, measurement of biochemical variables and safety parameters were conducted before randomization. During the two study visits, acylated ghrelin was measured in plasma samples taken before serving of the test drink and breakfast (baseline), at time 0, 20, 40, 80, and 180 min postprandially. The subjects had 15 min to finish the breakfast before the 0 min sample was taken. Blood pressure was measured before intervention, after 40 and 180 min after the intervention, as a safety parameter.

Appetite sensation was assessed on a Visual Analog Scale (VAS) of 100 mm in length, addressing the feeling of fullness and satiety. The VAS questionnaire also included three questions regarding adverse gastrointestinal symptoms (pain, discomfort, and nausea). The VAS was filled out five times during the study visit, at baseline, time 0 and 20, 40, and 180 min after the breakfast meal. Additionally, a questionnaire validated for the evaluation of different gastrointestinal symptoms was filled out before the breakfast meal and at the end of each study day (21). The questionnaire assessed nausea, bloating, stomach pain, constipation, and diarrhea, as well as hunger/satiety with a score from 0 to 10, of which the score 10 indicated severe symptoms and being fully satiated.

Test materials

The test material was a lemon-flavored powder provided from the manufacturer (Firmenich Bjørge Biomarin AS, Ålesund, Norway) in standardized bottles to be added 150 mL cold water. The powder contained 4% protein (CPH raw material or whole casein) and 96% carbohydrate (maltodextrin). Thorough laboratory tests assured that it was not possible to identify the active ingredient from the control, according to flavor or appearance. Each subject was given individually adjusted doses of 20 mg/kg body weight of CPH or casein. The drinks were made isonitrogenous to avoid bias due to difference in nitrogen content, and equal amounts of nitrogen in the form of casein were added to the control drink. Both drinks contained on average 1.6 g protein; thus it constituted only a small fraction of the total protein content of the standardized breakfast meal. Casein was chosen as the control as it has previously shown to not affect blood glucose or insulin sensitivity when compared with proteins from cod and soya (22). The casein used as control was present as whole protein and did not contain free amino acids or peptides. The production and composition of the test materials has been described in detail in previous publication (14).

Analysis of blood samples

Samples of venous blood were repeatedly collected using an intravenous catheter from the antecubital vein. Samples were analyzed according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital (Bergen, Norway) and the Department of Medical Biochemistry, Ålesund Hospital (Ålesund, Norway).

Plasma ghrelin was obtained by centrifugation of full blood using 4 mL anticoagulant (EDTA-K3)/Aprotinin blood collecting tubes (VACUETTE®, Greiner Bio One International GmbH, cat # 454261, Kremsmünster, Austria) at 1800 × *g* at 4°C for 10 min within 20 min after blood sampling. Plasma was stored frozen at −80°C prior to analysis. The ghrelin analyses were performed using Acylated Ghrelin-Easy Sampling Enzyme Immunoassay kit (Bertin Pharma, Montigny-le-Bretonneux, France, ref: #A11306).

Statistical analysis

SPSS data package (SPSS Statistics 24.0, IBM Company, Armonk, NY, USA) and GraphPad Prism version 7.0 (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical analysis. Shapiro–Wilk's test was conducted to assess normal distribution of data. A multivariable, repeated-measures linear mixed-effects regression analysis (adjusted for BMI and gender) was conducted in SPSS in order to evaluate the difference between the concentrations of acylated ghrelin after CPH and control. The data for acylated ghrelin were non-normally distributed; thus, it was log-transformed before analysis and presented as log mean and back-transformed values (geometric means). Graphical work and total area under the curve (tAUC) analysis for acylated ghrelin concentrations and symptom VAS-scores were conducted in GraphPad. The difference in baseline and end-point scores for the additional questionnaire was evaluated by a paired *t*-test. Assessment of correlations was done with Pearson's correlation coefficient. *P*-values < 0.05 were considered statistically significant.

The number of participants was not calculated according to a power analysis, due to lack of similar studies. Previous research reporting on effect of cod proteins in humans is based on whole fish (23) or long-term use of fish protein supplement (12, 13); thus, we did not find any data suitable as basis for power analysis for our design.

Results

Subjects

Seventy-eight subjects were screened for inclusion and exclusion criteria over telephone, of which 47 were enrolled to a screening visit. Six participants withdrew before the first study visit, and 41 participants completed the trial, of which 15 males and 26 females. The inclusion process has previously been described and illustrated (14). The mean age of the participants was 51 ± 6 years (range 40–64 years). The mean body weight of the participants was 77.3 ± 13.5 kg. Dependent on body weight, the subjects consumed CPH in a dose ranging from 1.2 to 2.3 g (mean 1.5 g). Mean BMI was 25.2 ± 3 kg/m^2. Twenty-three participants had BMI ≤ 25 kg/m^2, whereas 18 participants had BMI > 25 kg/m^2. Baseline characteristics and comparison of gender distribution are presented in Table 1.

Acylated ghrelin concentrations

Mean fasting acylated ghrelin levels were higher before the CPH intervention than before the control intervention (97.4 ± 196.3 pg/mL vs. 90.0 ± 194.0 pg/mL, respectively), but the difference was not significant ($P > 0.999$). Suppression of acylated ghrelin (C_{min}) was greatest: 80 min postprandially after CPH and 20 min postprandially after control, with a C_{min} mean difference from baseline of −14.0 ± 21.4 after CPH and −5.3 ± 22.8 after control, at these times, respectively ($P = 0.681$).

Statistically, no differences were observed between CPH and control for postprandial acylated ghrelin concentration in a mixed-effects regression analysis (mean difference of the geometric mean: 1.05 pg/mL, 95% confidence interval [CI]: 0.97–1.13, $P = 0.266$) (Fig. 1). Additionally,

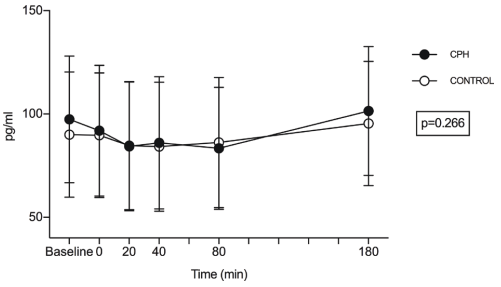

Fig. 1. Metabolic response in acylated ghrelin concentration after intake of a standardized breakfast meal supplemented with a drink containing a cod protein hydrolysate (CPH) or control (casein). Results are presented for 41 healthy subjects. Time point 0 min shows values measured right after the intake of breakfast and test drink. Values are presented as mean + SD. Statistically, no differences were observed between CPH and control for acylated ghrelin concentration in a mixed-model regression analysis ($P = 0.266$).

Table 1. Baseline characteristics of the 41 participants (26 females and 15 males) included in the study at the Haukeland University Hospital and Ålesund Hospital

Characteristics	Total subjects (*n* = 41)		Female (*n* = 26)		Male (*n* = 15)		*P*
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	
Age, years	51.0	6.0	52.1	6.2	49.0	5.0	0.104
Body weight	77.3	13.5	71.6	10.8	87.2	12.3	0.001
Body mass index, kg/m^2	25.2	3.0	24.7	3.0	26.0	2.9	0.183
Acylated ghrelin, pg/mL	93.7	194.9	81.4	184.7	115.0	210.6	0.453

Baseline acylated ghrelin concentrations are merged values for the baseline value at both study visits.

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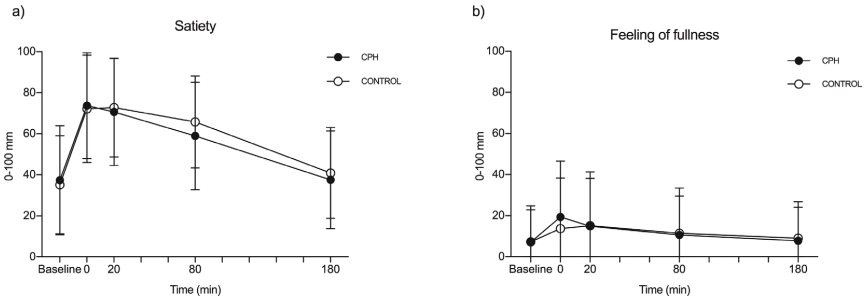

Fig. 2. Symptom scores from a VAS-questionnaire addressing satiety (a) and the feeling of fullness (b) after intake of a standardized breakfast meal supplemented with a drink containing either a cod protein hydrolysate (CPH) or control (casein). Results are presented for 41 healthy subjects. Time point 0 min shows values measured right after the intake of breakfast and test material. Values are presented as mean + SD. Statistically, no differences were found between CPH and control for sensation of appetite, according to the tAUC of postprandial scores for satiety ($P = 0.794$) and the feeling of fullness ($P = 0.966$).

no difference was observed between the tAUC for acylated ghrelin concentrations after CPH (tAUC = 17518 pg/mL × min, 95% CI: 0–47941) and control (tAUC = 17272 pg/mL × min, 95% CI: 0–48048, $P = 0.991$).

No correlation was observed between body weight (kg) and baseline concentration of acylated ghrelin (mean baseline value before CPH and control) ($r = 0.118$, $P = 0.463$). When adjusting for BMI and gender in the mixed-effects regression analysis, no differences were observed between subjects with BMI ≤ 25 kg/m^2 compared to those with BMI > 25 kg/m^2 ($P = 0.681$), or between genders ($P = 0.627$).

Sensation of appetite

Baseline scores for satiety were numerically the same before each intervention (CPH: 37.4 ± 26.6, control: 35.1 ± 23.9, $P = 0.997$). No difference was observed between the tAUC for the postprandial satiety scores after CPH (tAUC = 10989 mm × min, 95% CI: 6794–15185) and control (tAUC = 11742 mm × min, 95% CI: 8001–15483, $P = 0.794$). Data are presented in Fig. 2a.

Baseline scores for the feeling of fullness were numerically the same before each intervention (CPH: 7.1 ± 15.7, control: 7.3 ± 17.5, $P > 0.999$). No difference was observed between the tAUC for the postprandial feeling of fullness scores after CPH (tAUC = 1306 mm × min, 95% CI: 0–3257) and control (tAUC = 1243 mm × min, 95% CI: 0–3418, $P = 0.966$). Data are presented in Fig. 2b.

The questionnaire addressing hunger did not reveal any differences in the feeling of satiety and hunger between CPH (3.4 ± 1.8) and control (3.4 ± 2.3) 180 min after the breakfast ($P = 0.822$). Baseline scores did not differ before each intervention (CPH: 3.7 ± 2.7 and control: 3.0 ± 2.5, $P = 0.165$).

Gastrointestinal symptoms

There were no reports of adverse gastrointestinal symptoms (e.g. nausea, bloating, stomach pain, constipation, or diarrhea) during the exposure for either CPH or control.

Discussion

This study revealed no differences in postprandial concentrations of acylated ghrelin after a meal supplemented with CPH compared to control. Thus, we were unable to confirm our hypothesis that a single dose of CPH supplementation before a meal would suppress ghrelin concentrations postprandially, and thereby cause reduced feeling of hunger. Moreover, we found no differences between control and CPH drink in the feeling of satiety or feeling of fullness, as measured by the implemented questionnaires.

In a previous publication, we reported that supplementation with 20 mg CPH per kg body weight before a breakfast meal reduced the postprandial concentrations of insulin compared to control in healthy individuals (14). Although not affecting glucose levels or concentrations of GLP-1, we found that pre-prandial supplementation with one low dose of CPH may beneficially alter the glucose metabolism. An inverse correlation between postprandial insulin concentrations and plasma ghrelin has previously been reported (24), and changes in ghrelin concentration after CPH might affect postprandial insulin secretion. The major effects of ghrelin are linked to mechanisms involved in avoiding starvation and promoting food intake and include stimulation of GH secretion to restrict peripheral glucose uptake, promote lipolysis, and suppress insulin secretion to prevent hypoglycemia (25). Due to this close link between glucose metabolism and appetite control, it can be hypothesized that supplementation with CPH might influence

postprandial ghrelin concentrations and the feeling of hunger and satiety.

Our results are partly in line with previous similar single-dose studies; however, few studies are comparable in test material. Most studies investigating the relationship between ghrelin levels after meals with different macronutrient composition have found that a high-protein meal causes the ghrelin levels to be suppressed for a longer time than after the intake of a meal high in carbohydrates (26–31). Furthermore, it causes higher satiety scores postprandially than a meal containing regular or low amount of protein (28, 32). The mechanisms suggested to facilitate these findings include slowing of gastric emptying, increase of plasma insulin, glucagon, ghrelin, CCK, gastric inhibitory polypeptide (GIP), and GLP-1 after a high-protein meal (33).

Only a few studies have reported on the specific acute postprandial effect of a meal with proteins from fish, compared to other protein sources (34–36). A comparison of the effects of isocaloric meals with proteins from beef, chicken, or fish revealed a significantly higher satiety score after the fish meal compared to the other protein sources (34). A study evaluating the effect on satiety when comparing a fish protein meal with a beef protein meal revealed that subjects receiving the fish-meal had lower hunger scores and consumed less energy in the subsequent evening meal (36). In contrast, a study investigating the acute effect of meals based on proteins from cod or veal in combination with carbohydrates high- or low-glycemic index did not find any differences in appetite sensation, energy intake, or postprandial response in glucose, insulin, or ghrelin levels when comparing the two different protein sources (35). Although some previous studies have reported fish proteins to suppress appetite, no effect has this far been reported for the levels of ghrelin. One previous study has reported on the specific hunger-regulating effect of a fish protein hydrolysate from blue whiting (2 g/day) (17). The fish protein hydrolysate was reported to suppress appetite when compared to placebo in a 2-week crossover trial in overweight women. According to postprandial measures after a standardized breakfast meal, it was observed that the fish protein hydrolysate significantly reduced sweet-cravings, as well as plasma glucose levels compared to placebo. This study was based on observations made in both *in vitro* and *in vivo* models, showing that the fish protein hydrolysate was capable of enhancing the secretion of CCK and GLP-1, both hormones contributing to the regulation of energy intake (37).

In the present study, we hypothesized that a potential suppressing effect on appetite and postprandial ghrelin levels would occur due to the CPH containing a high fraction of di- and tripeptides with the branched-chain amino acids leucine and isoleucine. We hypothesize that these peptides work as biologically active substances, which are rapidly absorbed from the gastrointestinal tract and possibly capable of influencing pathways involved in the regulation of appetite. Thus, a single low dose of peptides was administered to the participants. The amount of protein provided was so low that it can be regarded negligible *per se,* compared to the amount of protein provided in the breakfast meal. Several factors could explain the lack of observed effect. First, it is possible that one acute exposure of the low concentration is not enough to induce the wanted effect. A different effect could might have been observed if the participants had taken the CPH supplement daily for a period of time, for instance over a period of 6–8 weeks.

A similar dose of CPH as the one administered in our trial, has previously been reported to increase concentrations of CCK and GLP-1 compared to placebo in a study including 120 overweight individuals given either 1.4 or 2.8 g protein hydrolysate from blue whiting or placebo, for 90 days (18). The fish protein hydrolysate was found to be effective compared to placebo, but no difference in effect was observed between the two doses. This demonstrates a potential effect when CPH is administered orally in doses of approximately 15–20 mg per kg body weight. In our study, the subjects consumed CPH in a dose range from 1.2 to 2.3 g (mean 1.5 g), dependent on body weight. Thus, if an effect was to be observed, this could possibly have been attributed to the presence of bioactive peptides.

The results have to be interpreted taking certain strengths and limitations into account. First, the randomized, crossover design as well as the successful blinding with similar test drink and control is a strength of this study. Furthermore, the adjustment of peptide dose according to the body weight of each participant can be regarded as an improvement in accuracy compared to previous investigations of protein meals and the few studies investigating a marine protein hydrolysate, as this may reduce the effect of variation in body weight. It can be regarded a weakness of the design that the control drink contained casein in equal amounts as CPH, and that we did not include a true placebo without protein. However, as discussed above, the hypothesized effect is attributed to the presence of bioactive peptides and not protein *per se*. Whole casein was chosen as control, so the observed effect should not be simply due to differences in energy and nitrogen content. Casein has previously been shown to not affect glucose metabolism when given in low concentrations (38). We could arguably have investigated the effect of CPH on several different hunger-regulating hormones, for instance, CCK, which has been measured in few other studies investigating the effect of a fish protein hydrolysate on appetite (17, 18). The assessment of appetite could have been improved by using a more comprehensive instrument at all timepoints (39). However,

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the use of VAS ratings in the evaluation of appetite in healthy subjects is a previously validated method (40). An *ad libitum* lunch meal with subsequent calculations of actual energy intake after CPH and control could have been included for a better and more detailed investigation of appetite. Furthermore, it is possible that the lack of sufficient previous data to perform a power analysis could result in too few included participants to be able to observe an effect. We decided to include 40 participants, a number greater than or equal to previously reported studies on cod protein (12, 13, 23).

Further studies should aim to evaluate the impact of fish protein hydrolysates on different metabolic pathways involved in glucose metabolism and appetite control, such as regulation of different hunger-regulating hormones. In addition to ghrelin, insulin-like peptide 5 (INSL5) is quite recently suggested to be an orexigenic hormone influencing appetite and regulation of food intake (41). Thus, future studies should aim to evaluate the appetite-regulating effect of CPH on several hormones, including INSL5. Assessments of such response can arguably contribute to the expansion of knowledge on the effects of CPH, as well as possibly reveal new preventive and treatment options for overweight and obesity. Based on the current literature, the effect could be more apparent if the fish protein hydrolysate had been given daily over a period of time, and if it had been investigated in a target group of overweight and obese individuals.Thus, the design of future studies should take this into account.

In conclusion, we did not find any effect of a single dose of CPH on postprandial concentrations of acylated ghrelin, or sensations-related appetite in healthy individuals, when compared to control after a standardized breakfast meal.

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Founding

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Authors

HFD, CJ, TH, IB, JGH, DALH and GAL designed the present study. HFD and CJ conducted the research. IB analysed the ghrelin samples. HFD wrote the manuscript. EL obtained funding and provided administrative, technical and material support. All authors reviewed and approved the manuscript.

Disclosure

Einar Lied is professor emeritus at the University of Bergen, Bergen, Norway, and former Scientific Advisor in Firmenich Bjørge Biomarin AS (Ellingsøy, Ålesund, Norway), where he holds a royalty agreement. The other authors declare no conflict of interest.

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RESEARCH ARTICLE

Supplementation with cod protein hydrolysate in older adults: a dose range cross-over study

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Abstract

A large proportion of older adults are affected by impaired glucose metabolism. Previous studies with fish protein have reported improved glucose regulation in healthy adults, but the evidence in older adults is limited. Therefore, we wanted to assess the effect of increasing doses of a cod protein hydrolysate (CPH) on postprandial glucose metabolism in older adults. The study was a double-blind cross-over trial. Participants received four different doses (10, 20, 30 or 40 mg/kg body weight (BW)) of CPH daily for 1 week with 1-week washout periods in between. The primary outcome was postprandial response in glucose metabolism, measured by samples of serum glucose and insulin in 20 min intervals for 120 min. The secondary outcome was postprandial response in plasma glucagon-like peptide 1 (GLP-1). Thirty-one subjects aged 60–78 years were included in the study. In a mixed-model statistical analysis, no differences in estimated maximum value of glucose, insulin or GLP-1 were observed when comparing the lowest dose of CPH (10 mg/kg BW) with the higher doses (20, 30 or 40 mg/kg BW). The estimated maximum value of glucose was on average 0·28 mmol/l lower when the participants were given 40 mg/kg BW CPH compared with 10 mg/kg BW ($P = 0{\cdot}13$). The estimated maximum value of insulin was on average 5·14 mIU/l lower with 40 mg/kg BW of CPH compared with 10 mg/kg BW ($P = 0{\cdot}20$). Our findings suggest that serum glucose and insulin levels tend to decrease with increasing amounts of CPH. Due to preliminary findings, the results require further investigation.

Keywords: **Fish protein: Cod protein: Marine peptides: Marine protein hydrolysate: Glucose homeostasis**

The human body is dependent on a tight regulation of blood glucose levels to ensure normal function[1]. Blood glucose levels are regulated within a narrow range, and glucose homeostasis is maintained through an intricate network of hormones and neuropeptides that are released in the body[1,2]. With increasing age, glucose metabolism changes and a large proportion of older adults are affected by impaired glucose metabolism[3,4]. Since skeletal muscle is the major site for insulin-stimulated uptake of glucose[5,6], it has been suggested that low skeletal muscle mass observed in some older adults

Abbreviations: BW, body weight; CPH, cod protein hydrolysate; GLP-1, glucagon-like peptide 1.

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with reduced muscle mass and function might result in reduced capacity for glucose disposal[7]. Furthermore, higher fasting and postprandial values of glucose and insulin have been associated with lower muscle mass in older adults[8]. The gradual decline in muscle mass and function observed with increasing age[9–11] is a major threat to healthy ageing, and causes reduced mobility, increased disability, loss of independence and overall reduced quality of life[12,13].

Several previous intervention studies have reported improved insulin sensitivity[14,15] and glucose tolerance[14,16] in humans and rodents after supplementation with fish protein. Furthermore, 3-month supplementation with a daily dose of 1·4 g protein hydrolysate from blue whiting given to overweight adults increased blood concentrations of glucagon-like peptide 1 (GLP-1). No further effects were observed when the participants were given a higher dose of 2·8 g, which might indicate a plateau effect starting at 1·4 g[17]. GLP-1 is released from the enteroendocrine L-cells in response to food intake and lowers blood glucose levels by stimulating insulin secretion, suppressing glucagon secretion and slowing gastric emptying[18]. In general, fish protein and hydrolysates from fish protein have a well-balanced distribution of amino acids and should be considered a high-quality protein source, and there is an increasing amount of evidence supporting a favourable effect of these proteins on metabolic health[19].

The evidence of health effects of cod protein as a nutritional supplement is limited, and only a few studies in healthy and overweight adults have been conducted. A recent study reported that an 8-week supplementation with 6 g of residual material from cod (press-cake meal) in a group of overweight or obese adults resulted in decreased postprandial concentrations of serum NEFA, which might indicate an effect on markers for glucose regulation[20]. In addition, a small pilot study in overweight adults observed improved glucose regulation after daily supplementation with 2·5 g of protein from cod for 8 weeks[21]. No changes in insulin, insulin C-peptide or NEFA in serum were observed[21]. Furthermore, we recently demonstrated that supplementation with a single dose of 20 mg/kg body weight (BW) of a protein hydrolysate from cod, given before a breakfast meal, reduced postprandial insulin secretion in forty-one healthy adults between 41 and 64 years, when compared with control[22]. We did not observe any effects on postprandial blood glucose response or on the levels of GLP-1.

Based on current knowledge, it is of interest to further explore potential favourable effects of cod protein on parameters closely related to muscle health, including parameters of glucose metabolism in an older population. To our knowledge, no previous trial has evaluated the effect of increasing doses of a supplement with cod protein hydrolysate (CPH) on glucose metabolism in older adults. Therefore, the aim of the present study was to investigate the effect of supplementation with four different weight-adjusted doses of a CPH on postprandial glucose regulation in a group of older adults aged 60–80 years. Based on the results from the study, we hoped to create a basis for selecting an effective daily dose of CPH for further use in clinical study protocols in patient groups with muscle health issues, inflammatory conditions or abnormal glucose metabolism.

Experimental methods

Study design

The study was a double-blind cross-over trial. The participants received four different doses (10, 20, 30 or 40 mg/kg BW) of CPH daily for 1 week with 1-week washout periods in between the dose intervals. Each participant received all four different dose intervals in random order. The participants were instructed to take the supplement each morning before breakfast for 7 d. After an initial screening visit, included participants came to the research unit on four different occasions, separated by 2 weeks. In total, the study lasted for 7 weeks.

The primary outcome was postprandial response in glucose metabolism, measured by venous samples of glucose and insulin. Secondary outcomes were plasma GLP-1 and adverse effects measured by symptom questionnaires.

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 Committee for Medical and Health Research Ethics of Central Norway (2017/1795). Written informed consent was obtained from all subjects. The trial is registered at www.clinicaltrials.gov as NCT03526744.

Participants

Participants were recruited by advertisement on the external websites and on notice boards at Haukeland University Hospital, Bergen and Ålesund Hospital, Ålesund. Recruitment took place between March and July 2018, and the study was conducted between April and November of the same year.

Potential participants were screened for general eligibility by telephone, and suitable candidates were invited for a baseline evaluation visit, with further information and baseline blood chemistry. The criteria for inclusion were age between 60 and 80 years, BMI between 20 and 30 kg/m^2 and signed informed consent. Criteria for exclusion were allergy and intolerances to fish and/or shellfish, pharmacologically treated diabetes mellitus, low or unstable blood pressure, chronic diseases or medication that were likely to interfere with the evaluation of the study endpoints, acute infections, substance misuse (excessive alcohol consumption and/or narcotic substances assessed by physician) or unwillingness to comply with the requirements of the study. The participants were instructed to not take any nutritional supplements containing *n*-3 PUFA for 2 weeks before study commencement and during the course of the study.

Study protocol

The participants came to the research unit on five different occasions, including a screening visit. Before inclusion, the subjects underwent clinical examination by a physician, baseline biochemistry and measurement of height, weight and blood pressure.

A 3-d prospective diet diary was recorded prior to starting the intervention, and at the end of the study period. The

level of physical activity was assessed at baseline and at the end of the study by asking the participants two questions regarding moderate physical activity and vigorous activity (self-reported). The participants were instructed not to change diet habits or the level of physical activity during the study period.

The study consisted of four different intervention cycles. Before each intervention cycle, the participants received six bottles containing powder with CPH, labelled 1 to 6. We instructed the participants to take one bottle each morning during the intervention cycle. On days of study tests, day 7 in each intervention cycle, the participants came to the research facility in a fasting condition between 08.00 and 09.00 hours. After baseline blood sampling, we gave the last dosage of CPH followed by a standardised breakfast meal 10 min later. At 25 min after the CPH drink was served and 15 min after the breakfast meal had started, we took the first postprandial blood sample (0 min sample). Thereafter, the participants spent 2 h in the vicinity of the research unit to allow for repeated sampling of blood, at 20 min intervals until 120 min.

The standardised breakfast meal consisted of two slices of semi-coarse bread (50 % whole wheat, 80 g bread), 10 g margarine, 20 g strawberry jam and 20 g white cheese, providing a total of 1485 kJ (355 kcal), 41 g carbohydrate, 12·5 g protein and 15 g fat. The drink contained 22·5 g carbohydrate and approximately 418 kJ (100 kcal), and including the drink, the breakfast provided in total 1900 kJ (455 kcal). The amount of energy and carbohydrates in the breakfast was calculated to induce an adequate blood glucose response. No coffee or tea was served, but water was given *ad libitum*.

We handed out the six bottles for the next intervention cycle at the end of the test day and gave instructions for when to start the next intervention cycle. Between intervention cycles, the participants had a washout period of 7 d. All participants received a text message on the morning of the day they were to start the next intervention cycle.

Assessments

At the screening visit we assessed the participant's medical history and measured biochemical parameters for nutritional status (albumin, prealbumin, vitamins B_{12} and D). We measured biochemical safety parameters at the screening visit and the end of study visit.

During the test days, baseline fasting serum glucose and serum insulin were measured 25 min before the first postprandial blood sample (time (t) = 0 min postprandial). Subsequently, serum glucose and insulin were measured every 20 min for 2 h (t = 20, 40, 60, 80, 100 and 120 min postprandial). Baseline GLP-1 was measured and thereafter postprandially at t = 0, 20, 40, 80 and 120 min. Blood pressure was measured at three time points during the test day as a safety parameter (t = 0, 40 and 120 min).

Two questionnaires evaluating the participants' symptoms were used to identify possible adverse events during each intervention period and on study visits. In each intervention period, a visual analogue scale was filled out before the participants took the first dose with CPH on day 1 and before the last dose on day 7. Further, a questionnaire validated for the evaluation of gastrointestinal symptoms[23] was filled out 2 h after intake of CPH on day 1 and day 7 (end of test day, t = 120 min).

Test material

The protein hydrolysate powder was delivered from the manufacturer (Firmenich Bjørge Biomarin AS) in neutral bottles coded with participant number and dose level (1–4). The bottles were coded by a person not involved in the performance of the study and the different dose levels were randomly allocated to the participants according to a central digital randomisation list. Study participants and investigators were blinded to the dose content in the bottles (double-blinded study). The key of randomisation was provided to the investigators when the trial had ended, and the statistical analysis was completed. The powder contained 4 % protein (CPH raw material) and 96 % carbohydrate (maltodextrin) and was flavoured with lemon. The CPH raw material contained approximately 89 % protein by weight, <0·2 % fat, 0 % carbohydrate, <3·0 % water, 10 % ash, 0·1 % NaCl, 1·7 % Na and 0·07 % chloride. The amino acid composition of CPH raw material is presented in Table 1. The hydrolysation process has been presented in a previous publication[22].

Estimation of energy intake

Calculations of energy and macronutrient intake were performed using *Kostholdsplanleggeren* (Norwegian Food Safety Authority and The Norwegian Directorate of Health, Oslo, Norway)[24], based on the reported food and drink intake data from the participants at baseline and at the end of the study. Participants registered their intake of food and drink

Table 1. Amino acid and taurine composition of the cod protein hydrolysate used in the present study

Amino acid	Total amino acid (mg/g)
Alanine	47·8
Arginine	51·1
Aspartic acid	73·3
Asparagine	0·38
Glutamic acid	125·0
Glutamine	0·78
Glycine	50·9
Histidine	13·5
Hydroxyproline	1·0
Isoleucine*	30·1
Leucine*	60·3
Lysine*	71·3
Methionine	22·1
Phenylalanine	23·2
Proline	29·7
Serine	36·0
Taurine	6·6
Threonine	30·9
Tryptophan	6·0
Tyrosine	22·7
Valine	36·9

* Branched-chain amino acids.

for three consecutive days, preferably including one weekend day, prior to the first dose and at the end of the study. The dietary records were used to record the participants' diet patterns and to assess whether the participants made changes to their diets during the study period.

Analyses of blood samples

Baseline biochemistry was analysed according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital (Bergen, Norway) and the Department of Medical Biochemistry, Ålesund Hospital (Ålesund, Norway).

Glucose and insulin were measured in serum according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital (Bergen, Norway). Serum was obtained by centrifugation of full blood at 2000 ***g*** at room temperature (20°C) for 10 min after 30–60 min of coagulation, using serum separator cloth activator tubes. Samples were aliquoted and stored at −80°C prior to analysis.

Plasma for the determination of GLP-1 was obtained by centrifugation of EDTA full blood at 1800 ***g*** at −4°C for 10 min, within 20 min after blood sampling. Prior to sampling, to EDTA blood sampling tubes were added 10 µl dipeptidyl peptidase-4 inhibitor (DPP4-010; DRG Diagnostics) per ml EDTA blood. GLP-1 plasma was aliquoted and stored at −80°C prior to analysis. The GLP-1 analyses were performed using an ELISA kit from IBL International GmbH (GLP-1 (7–36) active ELISA, reference RE53121).

Statistical analysis

Statistical analyses were performed using Stata v15.1 (StataCorp LLC) and SPSS software (IBM SPSS Statistics 24). Graphical work was conducted in GraphPad Prism version 7.0 (GraphPad Software, Inc.). Data are presented as means and standard deviations for continuous variables, and frequencies and relative frequencies for categorical variables. To estimate the effect of dose we calculated the maximum observed value and the AUC for the time course of each outcome variable, for each combination of person and dose. We then fitted mixed models with the outcome measure (maximum value or AUC) as the dependent variable, fixed effects of dose and random intercepts across persons. Carry-over effects were assessed using a standard likelihood-ratio test to test for interaction between dose and ordering. Paired-samples *t* tests were used to compare changes in energy intake and macronutrient intake from baseline to the end of the study. *P* values <0·05 were considered statistically significant.

The sample size was not feasible to calculate for power analysis, due to lack of similar studies. Possible health effects of supplementation with residual material from cod as protein hydrolysate has previously not been studied in a group of older adults, and therefore we had no basis for calculating sample size. According to protocol, we intended to include thirty participants.

Results

Demographic characteristics

From April to June 2018 we screened fifty-one subjects for study participation and thirty-three were enrolled in the study (Fig. 1). Two of the included participants were excluded before the first test day due to difficulties with blood sampling. Overall, thirty-one subjects aged 60–78 years completed the trial (thirteen males and eighteen females). One participant had to be excluded on the final study day due to difficulties with blood sampling; therefore data on glucose, insulin and GLP-1 are only available for three of the dose levels. Four of the participants were excluded from the final statistical analysis of GLP-1 due to analytical errors. Baseline characteristics of the participants are presented in Table 2.

Energy and macronutrient intake

No statistically significant differences were observed in energy intake or macronutrient intake during the course of the study (Table 3). One participant did not fill out the 3-d food record at the end of the study. Based on the reported intake of protein from the food diaries at baseline and at the end of the study, an average intake of 1·2 g protein/kg BW at baseline was estimated and this did not change during the study period ($P = 0{\cdot}36$; estimated average intake at end of study 1·1 g protein/kg BW).

Postprandial measurements

In a mixed-model analysis, no statistically significant differences in estimated maximum value of glucose, insulin or GLP-1 were observed when comparing the lowest dose of 10 mg/kg BW of CPH with 20, 30 or 40 mg/kg BW (Table 4). The estimated maximum value of glucose was on average 0·28 mmol/l lower when the participants were given the highest dose of 40 mg/kg BW CPH compared with the lowest dose of 10 mg/kg BW ($P = 0{\cdot}13$). The estimated maximum value of insulin was on average 5·14 mIU/l lower after participants were given the highest dose of 40 mg/kg BW of CPH compared with the lowest dose of 10 mg/kg BW ($P = 0{\cdot}20$). The estimated maximum value of GLP-1 was on average 0·34 pmol/l lower when given the highest dose (40 mg/kg BW) compared with the lowest dose of CPH (10 mg/kg BW) ($P = 0{\cdot}48$). No carry-over effect was observed for glucose ($P = 0{\cdot}19$), insulin ($P = 0{\cdot}21$) or GLP-1 ($P = 0{\cdot}08$).

No statistically significant differences in AUC between the four different doses were observed for any of the outcome measures when comparing the lowest dose of 10 mg/kg BW of CPH with the higher doses of 20, 30 or 40 mg/kg BW. For glucose, AUC was calculated from $t =$ baseline until $t = 80$, excluding $t = 100$ and $t = 120$ (Fig. 2), based on the assumption that for the majority of individuals, glucose levels had returned to their baseline levels. The AUC for glucose was on average 1·16 mmol/l × min higher when given 20 mg/kg BW of CPH ($P = 0{\cdot}14$), on average 0·27 mmol/l × min higher when given 30 mg/kg BW ($P = 0{\cdot}73$) and on average 0·78

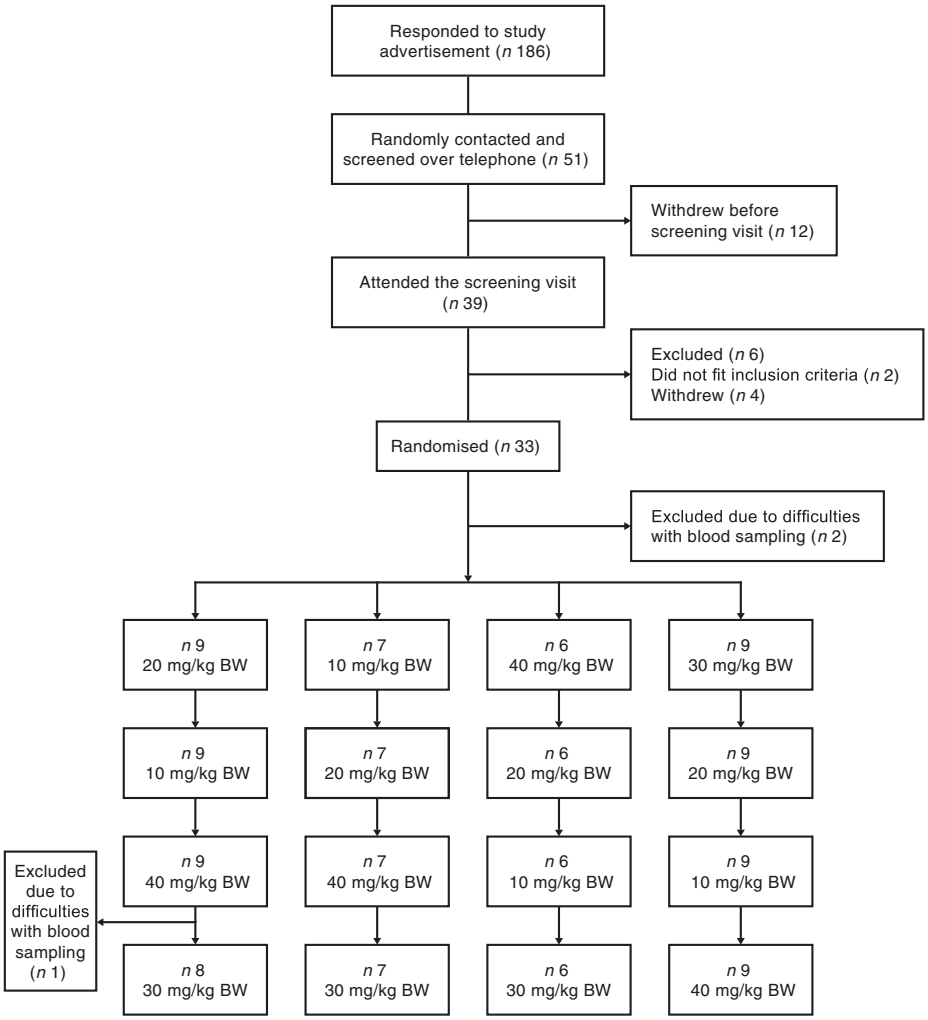

Fig. 1. Flowchart depicting the inclusion and randomisation process. BW, body weight.

Table 2. Baseline characteristics of the thirty-one participants (Mean values and standard deviations; numbers of subjects)

	Mean	SD
Subjects (*n*)	31	
Male	13	
Female	18	
Age (years)	67·8	4·9
Weight (kg)	76·6	11·3
BMI (kg/m^2)	26·0	2·6
Systolic BP (mmHg)	137	15
Diastolic BP (mmHg)	82	10
HbA1c (mmol/mol)	36·7	4·3
Fasting glucose (mmol/l)	5·4	0·6

BP, blood pressure; HbA1c, glycated Hb.

Table 3. Dietary intake at baseline and at the end of the study* (Mean values and standard deviations)

	Baseline		End of study		
	Mean	SD	Mean	SD	*P*†
Energy intake (kJ)	7765	1586	7807	1506	0·84
Protein (g)	86·9	20·6	84·2	18·1	0·32
Fat (g)	73·1	19·3	75·2	18·6	0·50
Carbohydrates (g)	215·2	65·4	213·8	55·8	0·95

* Food and drink intakes were registered for 3 d at baseline and at the end of the study.
† Paired-samples *t* tests were used to compare changes in energy intake and macronutrient intake from baseline to the end of the study. No significant differences were observed during the course of the study.

Table 4. Estimated maximum values of glucose, insulin and glucagon-like peptide 1 (GLP-1) derived from a mixed model (Mean differences and 95 % confidence intervals)

Outcome	Dose level (mg/kg BW)	Mean difference	95 % CI	*P*
Glucose (mmol/l)	10	0	(Reference)	
	20	0·33	−0·04, 0·70	0·08
	30	0·09	−0·29, 0·46	0·65
	40	−0·28	−0·65, 0·08	0·13
Insulin (mIU/l)	10	0	(Reference)	
	20	3·59	−4·34, 11·5	0·38
	30	3·42	−4·60, 11·4	0·40
	40	−5·14	−13·1, 2·79	0·20
GLP-1 (pmol/l)	10	0	(Reference)	
	20	−0·66	−1·59, 0·28	0·17
	30	−0·11	−1·06, 0·84	0·83
	40	−0·34	−1·28, 0·59	0·48

BW, body weight.

mmol/l × min lower when given 40 mg/kg BW ($P = 0·32$), when compared with the lowest dose of 10 mg/kg BW of CPH. If all measuring points were included in the statistical analysis of glucose, also including $t = 100$ and $t = 120$, the significance of the results did not change (Fig. 2). For insulin, the AUC was on average 11·3 mIU/l × min higher when given 20 mg/kg BW of CPH ($P = 0·49$), on average 6·84 mIU/l × min higher when given 30 mg/kg BW of CPH ($P = 0·67$) and on average 7·4 mIU/l × min lower when given 40 mg/kg BW ($P = 0·65$), when compared with the lowest dose of 10 mg/kg BW of CPH. For GLP-1, the AUC was on average 1·38 pmol/l × min lower when given 20 mg/kg BW of CPH ($P = 0·36$), on average 0·01 pmol/l × min lower when given 30 mg/kw BW of CPH ($P = 0·99$) and on average 1·09 pmol/l × min lower when given 40 mg/kg BW of CPH ($P = 0·47$), when compared with the lowest dose of 10 mg/kg BW of CPH. A graphical representation of the metabolic response for serum glucose, serum insulin and plasma GLP-1 concentration on the test day, the last day in the four different intervention cycles, is presented in Fig. 2. A bar chart showing total AUC for serum glucose, serum insulin and plasma GLP-1 is presented in Fig. 3.

Adverse effects

No adverse effects were reported in the questionnaires, from the biochemical safety parameters or from the biochemical parameters for nutritional status.

Discussion

The overall objective of the present study was to evaluate the effect of increasing doses of a supplement with CPH on glucose metabolism in older adults, aiming to find a dose response and creating a basis for an optimal daily dose for future clinical use. We investigated the effect on postprandial glucose regulation of four different doses of a CPH supplement (10, 20, 30 and 40 mg/kg BW) taken daily for 1 week. Although no statistically significant differences were observed between the postprandial measurements after the four different doses, our results indicate that the highest dose of CPH (40 mg/kg BW), equal to 3·2 g/d in an individual with a BW of 80 kg, is the most efficient in lowering postprandial blood glucose levels and insulin concentrations, when compared with the lower doses (10, 20 and 30 mg/kg BW).

In a previous publication, we reported that a single dose of 20 mg/kg BW CPH significantly reduced postprandial insulin concentrations in healthy, middle-aged to older individuals, without affecting postprandial glucose levels or GLP-1 levels, compared with control (casein)[22]. We hypothesised that the CPH might enhance the insulin sensitivity and affect other mechanism involved in blood glucose uptake in peripheral tissue. The significantly lower insulin concentration after intake of CPH may be of more interest in patients with reduced insulin sensitivity.

To our knowledge, only one small pilot study has been conducted with fish protein hydrolysate in an older population[25]. In this double-blind, randomised controlled study, a daily dietary supplement of 5·2 g fish protein hydrolysate from blue whiting, or placebo, was given to twenty-four nursing home residents daily for 6 weeks. No differences in serum concentrations of glucose or insulin after 6-week supplementation with fish protein were observed, when compared with placebo[25]. However, since this was a study population with

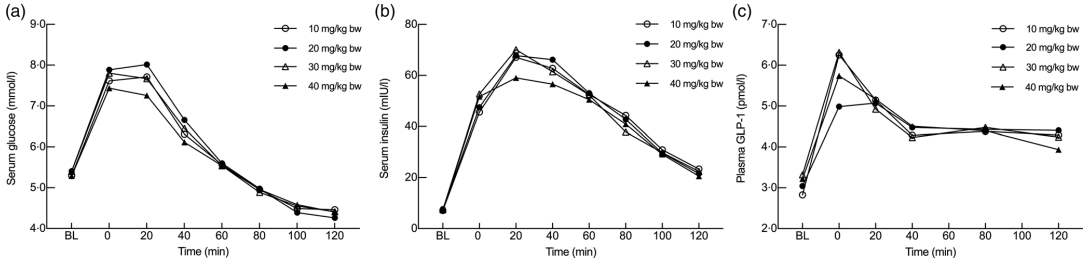

Fig. 2. Metabolic responses for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) concentrations after intake of a standardised breakfast meal and the last dosage of the cod protein hydrolysate (CPH). Dose levels were 10, 20, 30 and 40 mg/kg body weight (BW). Results for serum glucose and insulin are presented for all thirty-one subjects, whereas for GLP-1 the results are presented for twenty-seven subjects (four participants were excluded from the statistical analysis due to analytical errors). Values are means. Time point 0 min is the first postprandial blood sample, taken 25 min after the drink was served and 15 min after the breakfast meal started. BL, baseline.

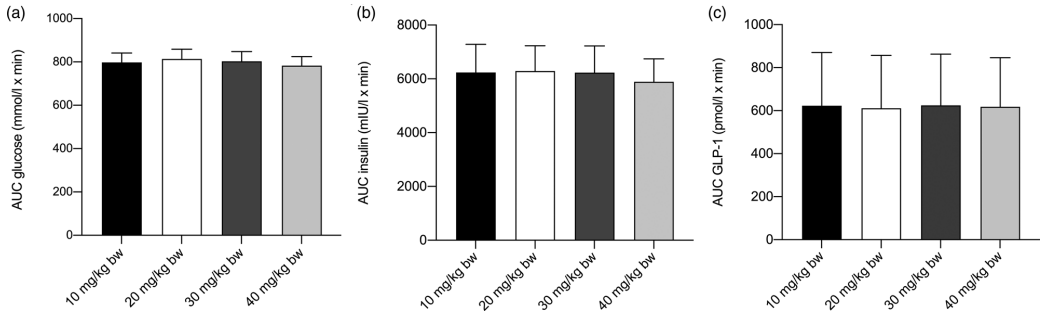

Fig. 3. Bar chart depicting the total AUC for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) after intake of a standardised breakfast meal and the last dosage of cod protein hydrolysate (CPH) in the dose level. Dose levels were 10, 20, 30 and 40 mg/kg body weight (BW). Values are means, with standard errors represented by vertical bars. No statistically significant differences in AUC between the four different doses were observed for any of the outcome measures when comparing the lowest dose of 10 mg/kg BW of CPH with the higher doses of 20, 30 or 40 mg/kg BW.

older adults who lived in a nursing home setting, the results are not directly transferable to our study population with home-dwelling older adults.

Based on a few previous studies investigating the effect of supplements containing protein hydrolysates from fish on metabolic health[17,26], we hypothesised that small doses of CPH may be effective due to the content of small, easily absorbable bioactive peptides. These are capable of rapidly affecting different metabolic pathways involved in glucose regulation and hence leading to a more rapid glucose response in the body. Thus, we presume that a potential observed effect on postprandial glucose metabolism can be attributed to the content of small, bioactive peptides in the supplement, and not the protein intake *per se*, which is negligible compared with overall protein content in a normal meal. Previous studies investigating supplements with fish protein or hydrolysates of fish proteins have reported doses in the range of 1 to 6 g per d to beneficially influence blood glucose metabolism when compared with control[16,17,21,26].

The results have to be interpreted taking certain limitations in the design into account. The use of a cross-over design always implies a risk of a carry-over-effect. According to analysis of all possible interaction effects between doses and time periods, the results in this cross-over trial are not biased by a carry-over effect. We included a washout period of 7 d between each week of peptide supplementation. We presume 1 week to be a sufficient washout period, as dietary protein in general has a high turnover rate and the investigated doses of protein hydrolysate were low[27]. On study days, the supplement was given to the participants 10 min before breakfast and 25 min before the first postprandial blood sample was taken. This design might have caused a metabolic response even before the breakfast was served. As a result, we may have missed some early information on postprandial glucose response. Furthermore, a 2-week washout period for the use of supplements containing *n*-3 PUFA before starting on the first dose of CPH may not have been enough and a longer washout period could arguably have strengthened the design. It is possible that the short supplementation period of 1 week could have affected the results, and that a longer period would have been preferable. However, we have previously observed an effect after only one acute supplementation (20 mg/kg BW) in healthy middle-aged adults[22]. A longer intervention period would have made it more challenging to include participants and avoid drop-outs, due to a long timeframe of the study. Therefore, due to practical implementations of the study, 1 week of supplementation (7 d) for each dose was chosen. Finally, the design could have been strengthened by including a postprandial blood sampling at day 0 for each intervention cycle or a control group (0 mg/kg BW CPH). However the study was performed based on a previous study, where we report that a low dose of CPH (20 mg/kg BW) significantly reduced the postprandial insulin concentration[22], and we therefore aimed to further evaluate the effect of different doses in the present study. An additional study day in each intervention period would also have made it more challenging to include participants and avoid a high drop-out rate, and would be difficult to implement due to limited resources. Based on this, we chose to only include postprandial blood sampling at the end of each intervention period.

To our knowledge, no previous publication has reported on the metabolic effect of different low doses of fish protein hydrolysate in an older adult population. Although no significant differences were observed in this trial, our findings suggest that low doses of fish protein hydrolysate might be effective and capable of improving blood glucose regulation in older adults. According to our findings, further studies investigating effects of supplements containing hydrolysates of fish proteins should be able to observe a metabolic effect from doses starting around 40 mg/kg BW, equal to 3·2 g per d in an individual with a BW of 80 kg. Based on this, we suggest that a dose ranging from 3 to 4 g per d is a reasonable starting point for future clinical studies. Due to preliminary findings, these results require further investigation.

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C. J., H. F. D., T. H., J. G. H., I. B., G. A. L. and D. A. L. H. designed the present study. C. J. and H. F. D. conducted the research. I. B. analysed the GLP-1 samples. C. J. and H. F. D. wrote the manuscript. E. L. obtained funding and provided administrative, technical and material support. All authors reviewed and approved the manuscript.

E. L. is former Scientific Advisor of Firmenich Bjørge Biomarin AS, Ellingsøy, Ålesund, Norway, where he holds a royalty agreement. The other authors declare no conflicts of interest.

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Article

Effects of a Cod Protein Hydrolysate Supplement on Symptoms, Gut Integrity Markers and Fecal Fermentation in Patients with Irritable Bowel Syndrome

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Abstract: Peptides from fish may beneficially affect several metabolic outcomes, including gut health and inflammation. The effect of fish peptides in subjects with irritable bowel syndrome (IBS) has not previously been investigated, hence this study aimed to evaluate the effect of a cod protein hydrolysate (CPH) supplement on symptom severity, gut integrity markers and fecal fermentation in IBS-patients. A double-blind, randomized parallel-intervention with six weeks of supplementation with 2.5 g CPH (n = 13) or placebo (n = 15) was conducted. The outcomes were evaluated at baseline and the end of the study. The primary outcomes were symptom severity evaluated by the IBS severity scoring system (IBS-SSS) and quality of life. The secondary outcomes included gut integrity markers and pro-inflammatory cytokines in serum, fecal fermentation measured by concentration of short-chain fatty acids (SCFAs) and fecal calprotectin. The groups were comparable at baseline. The total IBS-SSS-scores were reduced in both the CPH-group (298 ± 69 to 236 ± 106, p = 0.081) and the placebo-group (295 ± 107 to 202 ± 103, p = 0.005), but the end of study-scores did not differ (p = 0.395). The concentrations of serum markers and SCFAs did not change for any of the groups. The baseline measures for the whole group showed that the total SCFA concentrations were inversely correlated with the total IBS-SSS-score (r = −0.527, p = 0.004). Our study showed that a low dose of CPH taken daily by IBS-patients for six weeks did not affect symptom severity, gut integrity markers or fecal fermentation when compared to the placebo group.

Keywords: irritable bowel syndrome; bioactive fish peptide; short-chain fatty acids; low-grade inflammation; gut integrity markers

1. Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder affecting between 10–20% of the population [1], characterized by abdominal pain, bloating and/or distention, constipation and/or

diarrhea [2,3]. The pathophysiological mechanisms behind the condition are not fully understood but are suggested to include a combination of genetics, diet, abnormal gut microbiota, abnormal gut endocrine cells, increased intestinal permeability and low-grade inflammation [4,5].

Diet is considered as an important factor in IBS, with over half of the patients reporting worsening of symptoms in relation to intake of certain foods [6]. The effects of different sources of carbohydrates have been investigated in patients with IBS. Further, a diet low in fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) is currently one recommended dietary treatment [2]. FODMAPs are poorly digested in the small intestine. When reaching the colon fermentation of these carbohydrates by colonic bacteria, this leads to the generation of gases and short chain fatty acids (SCFAs). The levels of these metabolic end-products are altered in patients with IBS, conceivably causing symptoms [7,8]. The SCFAs exert several important physiological functions, such as influencing pathways involved in the gene regulation of the metabolism, inflammation and disease, as well as protect against diseases in the gastrointestinal tract [9]. A diet low in FODMAPs is associated with distinct alterations in the composition and function of the gut microbiota, and hence the levels of SCFAs. The long-term effect of the diet has not been well established [10,11].

Some dietary proteins are a source of bioactive peptides, exerting specific effects extending beyond the mere nutrient supply. These peptides can occur naturally by digestion in the gut or be consumed as already hydrolyzed proteins in dietary supplements [12]. Several animal studies have suggested that bioactive peptides from hydrolyzed fish proteins may beneficially influence health by improving the lipid profile, body composition and glucose metabolism [13–16]. This is supported by increasing evidence from recent clinical trials in human subjects, suggesting that supplements containing fish protein hydrolysates may beneficially influence several metabolic outcomes [17–21]. In addition, it is suggested that fish protein hydrolysates may have an immune-modulating effect with beneficial properties in the gut [22,23]. A chronic low-grade mucosal inflammation and increased intestinal permeability have been assumed to contribute to symptom generation in IBS patients, and several gut integrity markers have been investigated as potential biomarkers. These include zonulin, a physiologic regulator of intercellular tight junctions and suggested as a marker for impaired gut-barrier function [24]; lipopolysaccharide-binding protein (LBP), an acute phase protein suggested as a marker of bacterial translocation [25] and an intestinal fatty acid binding protein (iFABP), a marker for intestinal epithelial cell damage [26]. The clinical implications of these gut integrity markers in IBS have not been established. However based on the hypothesis of fish protein hydrolysate as a possible modulator of the gut, they can be relevant for evaluation in combination with pro-inflammatory cytokines.

The evidence and knowledge are limited on the specific impact of different sources of proteins in patients with IBS. According to clinical experience, IBS symptoms are most often linked to the digestion of carbohydrates, and further, dietary proteins are normally well tolerated. Investigations of different dietary sources of protein in healthy individuals have indicated that they affect the diversity and composition of the human gut microbiota in different degrees [27]. Recent results indicate that the presence of fish proteins in the diet have an impact on the composition and activity of the gut microbiome, influencing the microbiota composition [28]. To the authors' knowledge, the effect of a peptide supplement in IBS patients has previously not been reported. The environmental and economic benefits of expanding the utilization of by-products from the fishing industry, in addition to the need for novel, additional dietary treatment strategies for patients with IBS, make this study warranted.

The aim of this study was to evaluate the effect of a supplement with cod protein hydrolysate (CPH) on inflammation and gastrointestinal health, including changes in IBS symptoms. For this purpose, this study assessed symptom severity and analyzed gut integrity markers, including pro-inflammatory cytokines in serum, fecal fermentation products (SCFAs) and fecal calprotectin in patients with IBS.

2. Materials and Methods

2.1. Patients

The patients were recruited through advertisements on the internet between December 2018 and January 2019. The potential subjects answering the online recruitment form were interviewed for general eligibility and compliance with inclusion and exclusion criteria by telephone. The suitable candidates then received more information and signed the informed consent form. A 3-day dietary record and equipment to collect a baseline stool sample was sent by post to the participants prior to the baseline visit at the hospital.

The inclusion criteria were age 20–70 years, BMI 18–30 kg/m^2 and IBS diagnosis according to Rome IV criteria with predominant diarrhea (IBS-D) or mixed bowel movements (IBS-M). The exclusion criteria were fish allergy, diabetes mellitus, elevated blood pressure, chronic diseases (that might affect the evaluation of the study outcomes), acute infections, substance abuse, immunocompromised patients defined as taking immuno-suppressive medications, patients eating a strict low-FODMAP diet, use of antibiotics during the last 4 weeks before the inclusion or use of medications for the IBS diagnosis.

2.2. Study Design and Protocol

The study was a double-blinded, randomized parallel group trial, and included a six-week intervention with a drink containing 2.5 g CPH (test material) or 2.5 g maltodextrin (placebo). CPH or placebo powder was delivered to the patients in sealed bags containing doses for one day. The patients mixed the powder with water and drank it at least 10 min before breakfast each morning.

The baseline visit included assessment of medical data, IBS-diagnosis and biochemical variables. The Rome IV criteria were used to confirm the clinical diagnosis of IBS [29]. The study outcomes were evaluated at baseline and at the end of the study. The primary outcomes were symptom severity evaluated by IBS severity scoring system (IBS-SSS) and quality of life (QoL). The secondary outcomes included gut integrity markers and pro-inflammatory cytokines in serum, fecal fermentation measured by a concentration of short-chain fatty acids (SCFAs) and fecal calprotectin. All subjects completed a dietary record for three days prior to taking the fecal sample at baseline and at the end of the study. The subjects were instructed not to make any changes in the diet while attending the study, and not to take any nutritional supplements containing omega-3 or pre- or probiotics for 6 weeks before the study start, and during the study.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Regional Committees for Medical and Health Research Ethics of Central Norway (2018/1825) approved all procedures involving human. All subjects gave written informed consent and the trial was registered at clinicaltrials.gov as NCT03801057.

2.3. Test Material

The test material was a lemon-flavored powder provided from the manufacturer (Firmenich Bjørge Biomarin AS, Ålesund, Norway) in standardized sealed plastic-coated aluminum bags containing 8 g powder to be mixed with 100 mL cold water. Each powder bag contained 5 g glucose monohydrate, 0.0025 g Tastegram Powder Flavor, 0.1 g Lemongrass Durarome taste and 0.7 g citric acid, in addition to 2.5 g of CPH or maltodextrin (placebo). The thorough tests assured that it was not possible to identify the active ingredient from placebo, according to the flavor or the appearance.

The cod protein hydrolysate powder was made by Firmenich Bjørge Biomarin AS by hydrolyzing fish meat of Atlantic cod (*Gadus morhua*) with Protamex® (Novozymes AS) followed by spray drying of the soluble part of the enzyme digest. The CPH raw material contained approximately 89% protein by weight, <0.2% fat, 0% carbohydrate, <3.0% water, 10% ash, 0.1% NaCl, 1.7% sodium and 0.07% chloride. The free amino acids accounted for 4.77% of the total amino acids in the cod protein hydrolysate (CPH), and the ratio essential amino acids/non-essential amino acids was 0.70. The analysis of the molecular

weight distribution showed that approximately 90% of the peptides in the CPH had a molecular weight of 2000 Daltons (Da) or less (18 amino acids or less), approximately 75% of 1000 Da or less (10 amino acids or less), while approximately 55% had a molecular weight of 500 Da or less (5 amino acids or less). Approximately 25 to 30% of the peptides had a molecular weight less than 200 Da, which represents small dipeptides and free amino acids. The production process and composition of the CPH raw material has been described in detail in a previous publication [17].

The patients were randomly assigned to the experimental (CPH) or the control (placebo) group. Randomization was completed using a computer-based automated sequence implemented in the digital central case-report file (webCRF). The randomization sequence was generated by a person blinded to the assignment of patients to the study groups. The random assignment order was created using block randomization. The powder bag was coded by a person blinded to the allocation of patients. Both patients and study investigators were unaware of the study-group allocation (double-blinded study). The key of randomization was provided to the investigators when the trial had ended, and the statistical analysis was completed.

2.4. Blood Samples

The blood samples were taken at baseline and after the six-week intervention. General biochemical tests were taken for safety purposes (albumin, prealbumin, vitamin-B12, vitamin-D, leucocytes, thrombocytes, hemoglobin, $HbA1_c$, CRP, sodium, potassium, ALAT, ALP, creatinine and ASAT). The samples were analyzed according to standard accredited methods at the Laboratory for Clinical Biochemistry, Haukeland University Hospital and Department of Medical Biochemistry, Ålesund Hospital.

The gut integrity markers measured in the serum included iFABP, LBP and zonulin. In addition, this study analyzed the following pro-inflammatory cytokines in serum—tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ) and interleukins (IL-4, 6, 8, 10). The serum was obtained by centrifugation of full blood at 2000× *g* in room temperature (20 °C) for 10 min after 30–60 min of coagulation, using serum separator cloth activator tubes. The samples were aliquoted and stored at −80 °C until analysis. The analyses of cytokines were performed by a cytokine human ultrasensitive magnetic 10-plex panel for Luminex™ platform, Cat# LHC6004 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Furthermore, iFABP was analyzed by Human FABP2 (intestinal) ELISA kit Cat# EHFABP2 (Invitrogen, Thermo Scientific, Waltham, MA, USA), LBP was analyzed by Human LBP (Lipopolysaccharide-binding protein) ELISA kit, Cat# EKH3120 (Biosite, Taby, Sweeden) and zonulin was analyzed by IDK® Zonulin ELISA, Ref# K5601 (Immun diagnostic, Bensheim, Germany).

2.5. Fecal Samples

The fecal samples for analyses of calprotectin and SCFAs were collected before and after the six-week intervention. The patients were instructed to freeze the samples immediately after collection at home (−20 °C freezer) and bring the samples frozen to the hospital visits. The samples were stored at −20 °C until analysis.

The fecal samples for evaluation of calprotectin were collected in Calpro Easy Extract containers (Calpro AS, Oslo, Norway) and calprotectin content was measured using CALPROLAB™ Calprotectin ELISA (ALP) CALP0170 (CALPROLAB, Calpro AS, Oslo, Norway).

The fecal samples for evaluation of SCFAs were collected in designated containers (Sarstedt AG & Co., product No. 80.734.301, Numbrecht, Germany). Upon analysis, 0.5 g of the fecal material was added to distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4. 2.5 mL and then homogenized. After homogenization, 2.5 mL of the sample was vacuum distilled, according to the method of Zijlstra et al. [30], as modified by Hoverstad et al. [31]. The distillate was analyzed with gas chromatography (Agilent 7890 A, Calif., USA), using a capillary column (serial No. USE400345H, Agilent J&W GC Columns, Calif., USA) and quantified using internal standardization. Flame ionization detection was employed. The fecal samples were analyzed for both

major SCFAs (acetic, propionic and butyric) and minor SCFAs (iso-butyric, valeric, iso-valeric, capronic and iso-capronic acids). The results were expressed in mmol/kg wet weight.

2.6. Symptom Questionnaires

The symptoms related to the IBS diagnosis were assessed by symptom questionnaires at baseline and after the six-week intervention. The severity of abdominal symptoms was assessed by the validated IBS severity scoring system (IBS-SSS). The maximum score is 500 points, with the following grading: Mild (75–175 points), moderate (175–300) and severe (>300 points). A reduction of 50 points or more in the IBS-SSS questionnaire is regarded as clinically relevant [32]. The Quality of life (QoL) was evaluated by the validated Short Form-Nepean Dyspepsia Index (SF-NDI) with a maximum sum score of 50 points [33].

2.7. Estimation of Nutritional Intake

The calculations of energy and macronutrient intake, as well as FODMAP content in the diet, were performed using the Nordic nutrient calculation program Dietist Net Pro (Bromma, Sweden). The estimations reported were the mean daily intake based on three days of dietary records registered at baseline and during the last days of the six-week intervention. The total FODMAP content is the sum of calculated fructose, fructose in excess of glucose, lactose, fructans, polyols, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS).

2.8. Statistical Analysis

The statistical analyses were conducted in SPSS data package (SPSS Statistics 24.0, IBM Company, Armonk, NY, USA) and GraphPad Prism version 7.0. (GraphPad Software, Inc., San Diego, CA, USA). For all graphical work, this study used GraphPad Prism. The data are presented as the mean ± SD, unless otherwise stated. To compare the differences between the baseline and the end of the study measures for each subject, paired *t*-tests, and unpaired *t*-tests were used comparing differences between the CPH and the placebo group. The assessment of correlations was completed with Pearson's correlation coefficient. All tests were two-sided and p-values < 0.05 were considered statistically significant.

A power calculation for estimation of the sample size was not performed. According to protocol, this study intended to include 30 patients.

3. Results

3.1. Patient Characteristics

Thirty-one eligible patients were included, of whom 28 patients (23 women and 5 men) completed the trial and were included in the analyses. Three patients withdrew after randomization, one patient due to disliking the supplement and two patients due to experiencing increased diarrhea. The inclusion process is showed in Figure 1. According to the Rome IV phenotype definition, 19 patients were classified as diarrhea-predominant (IBS-D) and 9 patients as mixed bowel habits (IBS-M). Ten patients reported to avoid specific high-FODMAP food items they experienced as problematic (e.g., lactose, apples, wheat and/or garlic). In accordance with the inclusion criteria, no subjects followed a strict low FODMAP diet. According to total IBS-SSS scores at baseline, IBS severity was classified as mild in 2 patients, moderate in 9 patients and severe in 17 patients. The groups were comparable at baseline, except for a significant difference in BMI. Table 1 elucidates the baseline characteristics.

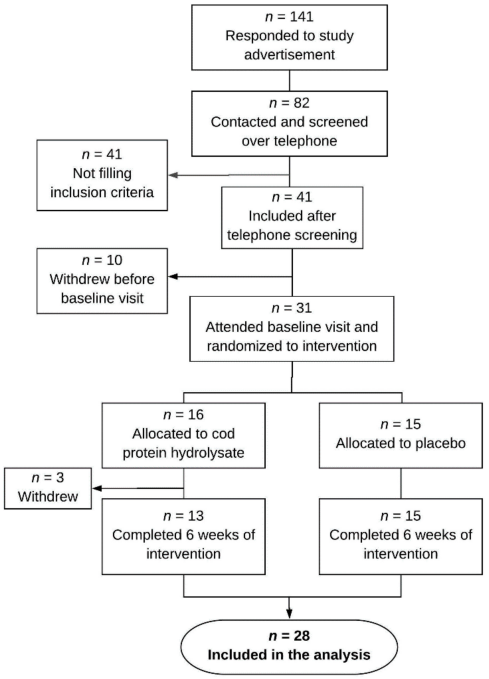

Figure 1. Flow-chart describing the inclusion process of the 28 irritable bowel syndrome (IBS) patients completing the six-week trial and included in the analysis.

Table 1. The baseline characteristics of 28 irritable bowel syndrome (IBS) patients randomly allocated to six weeks supplementation with either cod protein hydrolysate (CPH) or placebo.

Characteristics	CPH (n = 13)	Placebo (n = 15)	p-Value
Age, years	42.7 (11.9)	45.1 (14.8)	0.647
Gender (male/female)	1/12	4/11	-
BMI, kg/m^2	24.1 (2.8)	27.2 (3.9)	0.025 *
IBS-D/IBS-M	8/5	11/4	-
IBS severity [1] mild	0	2	-
moderate	5	4	-
severe	8	9	-
IBS-SSS sum score (0–500)	295 (107)	298 (69)	0.928
Energy intake, kcal/day	1750 (500)	1950 (395)	0.245
Protein intake, g/kg BW/day	1.2 (0.4)	1.0 (0.3)	0.185
Carbohydrates, g/day	140.0 (68.1)	180.2 (53.6)	0.093
Fiber, g/day	18.9 (7.6)	17.9 (5.7)	0.697
Total FODMAP [2], g/day	11.2 (6.6)	13.0 (11.3)	0.623
Alcohol, g/day	5.3 (6.8)	6.2 (8.6)	0.760
Fat, g/day	130.0 (190.5)	91.7 (22.6)	0.445

BW: body weight; BMI, Body Mass Index; IBS-D, IBS with diarrhea; IBS-M, mixed IBS; FODMAP, fermentable oligo-, di-, monosaccharides and polyol; IBS-SSS, IBS severity scoring system. [1] IBS severity based on the baseline IBS-SSS sum score: mild (75–175 points), moderate (175–300) and severe (>300 points); [2] Total FODMAP content in the diet based on mean daily intake from 3-days dietary records. * Statistically significant difference between the groups.

3.2. Irritable Bowel Syndrome Symptom Scores and Quality of Life

Table 2 reports symptom scores. According to total IBS-SSS scores, IBS symptoms improved from baseline to after six weeks of intervention in both the CPH-group (from 298 ± 69 to 236 ± 106, p = 0.081)

and the placebo-group (from 295 ± 107 to 202 ± 103, $p = 0.005$) (Figure 2). Regarding the mean difference from baseline to after the intervention, the total IBS-SSS score did not differ significantly between the CPH-group (−62 ± 118) and the placebo-group (−93 ± 108, $p = 0.471$) (Figure 3). After the intervention, the scores did not differ significantly between the groups ($p = 0.395$).

All IBS-SSS sub scores (pain severity, pain frequency, bloating, bowel habit dissatisfaction and life interference) declined from baseline to the end of the study in both groups. For the placebo-group, all symptoms declined significantly, whereas for the CPH-group, only bloating and life interference significantly declined. Significant differences between the groups for any other of the reported symptoms, either at baseline or at the end of the study, did not occur. The baseline measures for the whole group ($n = 28$) showed no significant correlations between total IBS-SSS scores and the calculated total FODMAP content in the diet.

The scores for QoL declined in both groups from baseline to the end of the study, with a significant reduction in the placebo-group (Table 2). The scores did not differ between the groups either at baseline ($p = 0.191$) or after the intervention ($p = 0.094$).

Table 2. Symptoms scores at baseline and after the six-week intervention for IBS patients in the cod protein hydrolysate (CPH) group and the placebo-group.

		CPH (n = 13)			Placebo (n = 15)		
Symptom Scores		**Baseline**	**End of Study**	***p*-Value**	**Baseline**	**End of Study**	***p*-Value**
IBS-SSS	Sum score	298.1 (68.9)	236.0 (105.9)	0.081	294.9 (106.6)	201.7 (103.6)	0.005 *
	Pain severity	45.0 (25.1)	39.2 (25.3)	0.096	43.3 (33.3)	25.0 (32.3)	0.016 *
	Pain frequency	45.4 (34.3)	39.2 (25.3)	0.446	47.3 (35.5)	25.3 (28.5)	0.018 *
	Bloating	65.9 (18.5)	46.0 (27.5)	0.046 *	59.3 (35.9)	37.0 (31.1)	0.038 *
	Bowel habit dissatisfaction	77.1 (20.1)	62.3 (30.8)	0.059	73.7 (26.2)	54.0 (30.1)	0.034 *
	Life interference	78.5 (17.1)	57.4 (30.4)	0.023 *	71.3 (23.6)	60.3 (22.2)	0.034 *
SF-NDI	Sum score	28.0 (7.1)	23.9 (9.1)	0.104	24.1 (7.9)	18.3 (7.9)	0.042 *

IBS-SSS: Irritable bowel syndrome severity scoring system, SF-NDI: Short Form-Nepean Dyspepsia Index, * Statistically significant difference between the baseline score the and end of study score.

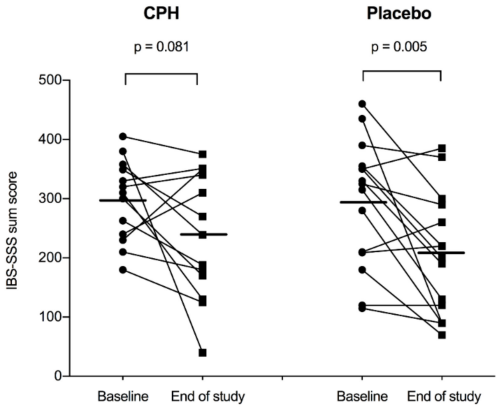

Figure 2. IBS-SSS scores at baseline and after the six-week intervention for the cod protein hydrolysate (CPH) group ($n = 13$) and the placebo-group ($n = 15$). The horizontal lines show the mean values.

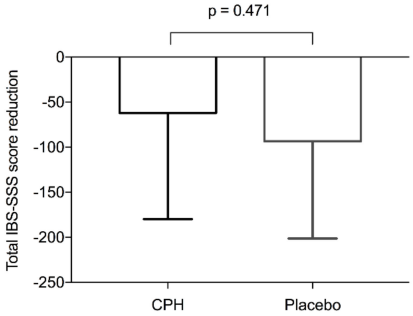

Figure 3. The reduction in total IBS-SSS scores from baseline to after the six-week intervention for the cod protein hydrolysate (CPH) group (n = 13) and the placebo-group (n = 15) expressed as the mean difference from baseline.

3.3. Gut Integrity Markers and Pro-Inflammatory Cytokines in Serum

The values for gut integrity markers and pro-inflammatory cytokines in serum are shown in Table 3. No significant changes in concentrations of LBP, iFABP or zonulin (ng/mL) were observed between baseline and the end of the study, in either the CPH-group or the placebo-group. The levels of zonulin were significantly lower for the CPH-group than the placebo-group at baseline (p = 0.011), but no other differences were observed between the groups. For the analyzed pro-inflammatory cytokines, only IL-8 showed values within detectable range, thus no data are reported for IL-4, 6 10, TNF-α and INF-γ. The concentration of IL-8 (pg/mL) increased from baseline to the end of the study in both groups, but the increase was not significant, and no differences were observed between the groups.

The baseline measures for the whole group (n = 28) showed no significant correlations between the serum markers and the total IBS-SSS score.

Table 3. The concentrations of gut integrity markers and pro-inflammatory cytokines in serum samples collected before and after six weeks of supplementation with cod protein hydrolysate (CPH) or placebo.

	CPH (n = 13)			Placebo (n = 15)		
Inflammatory Marker	**Baseline**	**End of Study**	***p*-Value**	**Baseline**	**End of Study**	***p*-Value**
iFABP (ng/mL)	68.3 (43.2)	58.2 (28.0)	0.432	55.5 (20.1)	56.2 (28.7)	0.940
LBP (ng/mL)	6097 (2630)	6446 (2043)	0.355	6931 (3023)	6884 (3274)	0.925
Zonulin (ng/mL)	40.5 (5.6)	42.5 (6.3)	0.125	46.6 (5.9)	45.7 (5.3)	0.286
IL-8 (pg/mL)	8.8 (11.8)	11.4 (10.1)	0.185	7.4 (6.5)	8.9 (9.1)	0.413

iFABP: Intestinal fatty acid binding protein, LBP: Lipopolysaccharide binding protein, IL: Interleukin.

3.4. Fecal SCFAs

This study observed no significant changes in concentrations of any SCFAs between the baseline and the end of the study measures, either in the CPH-group or the placebo-group. Table 4 outlines the values. No significant differences were observed between the groups for any of the measured SCFAs, either at baseline or the end of the study. The fecal total SCFA concentrations at baseline for the whole study population were inversely correlated with the IBS-SSS baseline sum score (r = −0.527, p = 0.004) (Figure 4). No correlations were observed between the total SCFA concentration and the serum markers or the total FODMAP content in the diet for the whole group at baseline.

Table 4. Short-chain fatty acid (SCFA) concentrations (mmol/kg) in fecal samples collected before and after six weeks of supplementation with cod protein hydrolysate (CPH) or the placebo.

	CPH (n = 13)			Placebo (n = 15)		
Parameter	**Baseline**	**End of Study**	***p*-Value**	**Baseline**	**End of Study**	***p*-Value**
Total SCFA	51.8 (22.4)	55.7 (24.1)	0.591	62.6 (19.5)	62.4 (23.1)	0.997
Acetic acid	30.4 (12.3)	32.2 (14.5)	0.705	36.3 (11.9)	35.9 (11.3)	0.921
Propionic acid	9.9 (6.3)	10.4 (6.7)	0.768	10.7 (3.8)	10.8 (6.0)	0.963
Butyric acid	7.4 (3.9)	8.5 (5.0)	0.473	10.3 (4.5)	10.0 (5.1)	0.827
Iso-butyric acid	1.1 (0.5)	1.3 (0.6)	0.257	1.3 (0.7)	1.4 (0.8)	0.595
Valeric acid	1.1 (0.8)	1.2 (0.6)	0.785	1.7 (0.9)	1.8 (1.0)	0.805
Iso-valeric acid	1.6 (0.8)	1.9 (1.0)	0.322	1.9 (1.1)	2.0 (1.3)	0.554
Caproic acid	0.3 (0.5)	0.3 (0.4)	0.992	0.5 (0.5)	0.6 (0.8)	0.425
Iso-caproic acid	0.0 (0,0)	0.0 (0.0)	-	0.01 (0.04)	0.01 (0.04)	0.670

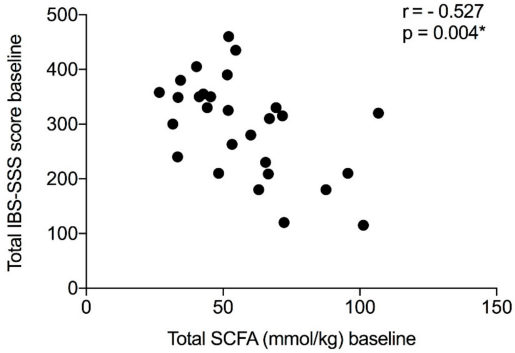

Figure 4. The relationship between the total concentration of SCFAs and the total IBS-SSS score at baseline for 28 patients with IBS. * The fecal total SCFA concentrations at baseline were statistically significant inversely correlated with the IBS-SSS baseline sum score.

3.5. Fecal Calprotectin

No significant changes in concentration of fecal calprotectin was observed between the baseline and the end of the study measures, either in the CPH-group (baseline: 137 ± 213 mg/kg, after intervention: 129 ± 134 mg/kg, p = 0.216) or the placebo-group (baseline: 117 ± 248, end of study: 99 ± 157, p = 0.525). Numerically, the mean value decreased slightly from baseline to the end of the study for both groups. No significant differences were observed between the groups either at baseline (p = 0.525) or at end of the study (p = 0.496).

3.6. Dietary Records

The comparison between the mean daily nutrient intake (kcal, proteins (g/kg body weight), carbohydrates (g), fiber (g), total FODMAPs (g), fat (g) and alcohol (g)) revealed no differences between the two groups either at baseline or at end of the study. No significant changes in nutrient intake from baseline to the end of the study were observed within each group.

3.7. Adverse Events

Three subjects allocated to CPH supplement withdrew after inclusion. One did not like the smell of the supplement, thus reported nausea associated to consumption. Two subjects reported an increase in IBS symptoms related to diarrhea and/or pain, of which one related the increase in symptoms to the supplement, whereas one acknowledged the symptoms as a regular bad IBS period.

4. Discussion

To the authors' knowledge, this is the first study reporting on the effects of a fish peptide supplement in IBS patients. The study was designed to investigate the effect of a dietary supplement with cod protein hydrolysate, hypothesized to contain bioactive peptides with potentially beneficial properties in the gut. This study observed no significant effects of the supplement for any of the outcome measures, when compared to the placebo.

The lack of effects could be explained by several factors. It was a reduction in symptom scores (primary outcome) during intervention in both the CPH and the placebo group, with a significant reduction in the total score only in the placebo group, and no significant differences between groups. Interventions in IBS patients are likely to be influenced by a strong placebo or nocebo effect [34]. As this study did not observe any changes in secondary outcomes to support an effect of the CPH supplement, it was assumed that the symptom reduction in both groups can be attributed a placebo effect, caused by the patients' expectations on symptom improvement when taking a dietary supplement in a clinical trial. It is possible that a different effect may have been observed if the hydrolysate was given in a higher dose. Previous studies investigating the health effects from a supplement containing hydrolyzed proteins from fish in human individuals have reported beneficial metabolic effects in a low dose range of 1 to 6 g a day [17–20]. Based on this, the authors chose an intervention with 2.5 g per day. This dose is negligible per se when put in context with the total daily dietary protein intake. Thus, if an effect were to be observed, our hypothesis was that it could have been attributed to the CPH.

According to our findings, no changes were observed in SCFA concentration after the intervention, but there was an inverse correlation between the total SCFA concentration at the baseline and the total IBS-SSS score when looking at the whole study population. This indicated that those with higher concentrations of SCFAs have less IBS-related symptoms. Previous studies investigating alterations in SCFA concentrations in IBS patients have reported inconsistent results. However, differences in fecal SCFA concentrations have been reported between IBS patients and healthy controls [35]. In addition, altered concentrations of both SCFAs and cytokines have been observed in response to a low-FODMAP diet in IBS patients [36,37]. The clinical relevance of a change in fecal SCFA concentration is currently not known. The fecal fermentation is dependent on both diet and gut microbiota, and the primary source for colonic production of SCFAs is low-digestible carbohydrates [37]. As the intake of carbohydrates did not change during the intervention, the lack of distinct findings in the current trial was not surprising.

The authors hypothesized that the CPH might influence inflammation and gut permeability, hence pro-inflammatory cytokines and gut integrity markers were evaluated. A change in either gut integrity markers or pro-inflammatory cytokines in response to intervention was not observed. Based on the theory of increased gut permeability and low-grade inflammation as a central contributor to IBS etiology, several studies have compared a broad range of gut integrity markers and inflammatory markers in IBS patients to healthy controls, aiming to identify possible biomarkers. To date, the findings have been inconsistent, but a reported tendency has been altered levels of gut integrity markers [24,26,38] and higher levels of pro-inflammatory cytokines [39–41] in IBS patients compared to healthy controls. Interestingly, of the analyzed cytokines, only IL-8 showed values within a detectable range at baseline, suggesting that neither IL-4, IL-6, IL-10, TNF-α or INF-γ are relevant as inflammatory markers for our IBS population with predominant diarrhea or mixed bowel habits. However, the measured levels of fecal calprotectin, with the mean baseline levels of above 100 mg/kg and levels above 50 mg/kg regarded as a positive value, support the assumption of low-grade inflammation as a contributor to disease. Hence, other inflammatory markers other than the cytokines investigated in this trial might be of interest in future studies.

The potential beneficial effects of dietary supplements with peptides and amino acids are in general not well investigated in IBS populations. Interestingly, Zhou et al. recently reported that dietary supplementation with the essential amino acid glutamine significantly improved symptoms in patients with post-infectious, diarrhea-predominant IBS [42]. Supplementation with glutamine (5 g per

day for eight weeks) was found to restore the intestinal permeability, leading to a reduction of diarrhea and abdominal pain, compared to the control (whey protein). Notably, the CPH used in our study holds a low concentration of glutamine, 0.78 mg/g CPH, corresponding to 1.95 mg glutamine per day with a dose of 2.5 g CPH (data on composition of the CPH are reported in a previous publication [17]).

The design holds some limitations. The cohort of the patients studied included only IBS-D and IBS-M subtypes. The absence of the effect of the intervention could be due to either the small cohort of the patients studied or to the low dose used. A larger cohort, including all the IBS subtypes, is needed before drawing any conclusion.

5. Conclusions

In summary, no effects of a supplement with 2.5 g CPH given daily for six-weeks was observed on symptom severity, gut integrity markers, pro-inflammatory cytokines or fecal fermentation products in a small group of patients with IBS, when compared to the placebo. Future studies should aim to target low-grade inflammation and evaluate the potential effect of supplementation with peptides containing bioactive sequences with known anti-inflammatory properties in IBS patients.

Author Contributions: Conceptualization, H.F.D., T.H., J.G.H., D.A.L.H. and G.A.L.; data curation, H.F.D.; formal analysis, H.F.D. and I.B.; investigation, H.F.D. and D.A.L.H.; project administration, H.F.D. and C.J.; supervision, T.H., J.G.H., D.A.L.H. and G.A.L.; validation, J.V., I.B. and G.A.L.; writing—original draft preparation, H.F.D.; writing—review and editing, C.J., T.H., J.G.H., I.B., J.V., D.A.L.H. and G.A.L.

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Conflicts of Interest: The authors declare no conflicts of interest.

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