Supplementation with cod protein hydrolysate

Intervention studies in healthy adults and adults with metabolic syndrome

Caroline Jensen

Thesis for the degree of Philosophiae Doctor (PhD)
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Main supervisor: Assoc. Professor, MD, Dag Arne Lihaug Hoff

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List of abbreviations

ACE	Angiotensin-1 converting enzyme
AKT	Protein kinase B
AUC	Area under the curve
ATP III	Adult Treatment Panel III
BCAA	Branched chain amino acid
BMI	Body mass index
BP	Blood pressure
BW	Body weight
CPH	Cod protein hydrolysate
CRP	C-reactive protein
CVD	Cardiovascular disease
DPP-4	Dipeptidyl peptidase-4
FFA	Free fatty acid
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT4	Glucose transporter type 4
HR	Heart rate
HbA1c	Glycated haemoglobin
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
IDF	International Diabetes Federation
IL	Interleukin
JIS	Joint Interim Statement

MetS	Metabolic syndrome
MCP-1	Monocyte chemoattractant protein-1
MW	Molecular weight
NCD	Non-communicable disease
NCEP	The National Cholesterol Education Program
OGTT	Oral glucose tolerance test
PEPT1	Peptide transporter 1
PI3K	Phosphatidylinositol 3-kinases
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trial
SDG	Sustainable Development Goals
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TNF-α	Tumor necrosis factor alpha
TOTAL C	Total cholesterol
VLDL-C	Very-low density lipoprotein cholesterol
WC	Waist circumference
WHO	World Health Organization

Abstract

Background: Metabolic syndrome (MetS) is a cluster of metabolic disturbances, including hyperglycaemia, hypertension, dyslipidaemia and abdominal obesity, increasing the risk of non-communicable diseases, such as cardiovascular disease and type 2 diabetes mellitus. Animal and clinical studies have observed favourable effects on glucose regulation and lipid metabolism after the intake of fish protein. As large amounts of residual raw materials are generated within the fishing industry every year, it is of interest to investigate if these already harvested marine resources may have similar beneficial metabolic effects in human.

Aim: To investigate the effect of supplementation of hydrolysed proteins, made from residual materials from cod fillet production, on glucose metabolism in healthy adults and components of MetS in overweight and obese adults.

Methods: Three clinical intervention studies were conducted. Study 1 was a randomised, double-blinded crossover trial in 41 healthy adults aged 40-65 years. The study included two study visits, with 4-7 days washout period in between the visits. The intervention was 20 mg/kg body weight (BW) of cod protein hydrolysate (CPH), or casein as control, given before a standardised breakfast meal. Study 2 was a dose-range crossover study in 31 adults aged 60-80 years old. The participants received four different weight-adjusted doses of 10, 20, 30 or 40 mg/kg BW of CPH in random order. Each dose level was taken daily for one week, with seven days washout period between different dose levels. The primary outcome of Study 1 and 2 was postprandial response in glucose metabolism, measured by serum samples of glucose, insulin and plasma glucagon-like peptide 1 (GLP-1) (Papers I and II). Study 3 was a randomised, double-blinded placebo-controlled trial in 30 overweight or obese adults with MetS. The participants received a once-daily supplementation of 4 g of CPH (n=15) or placebo (n=15) for 8 weeks. The primary outcomes were fasting and postprandial response in glucose. Secondary outcomes were changes in other components of the MetS, body composition, total serum cholesterol, insulin, GLP-1, ghrelin, inflammatory markers and adipokines (Papers III and IV).

Results: The postprandial insulin concentration was statistically significantly lower when subjects were given one single dose of CPH compared to control, with no statistically significant differences observed for postprandial concentrations of glucose or GLP-1 (Paper I). We observed no differences in estimated maximum value of glucose, insulin or GLP-1 when comparing the lowest dose of 10 mg/kg BW of CPH with the higher doses (20, 30 or 40 mg/kg BW) (Paper II). In subjects with MetS, supplementation with CPH for 8 weeks had no effect on postprandial glucose, insulin or GLP-1, lipid profile or body composition, when compared to placebo (Paper III). Furthermore, no effect on circulating levels of ghrelin, adiponectin, leptin or inflammatory markers were observed after CPH supplementation (Paper IV).

Conclusion: A single dose of CPH given before a standardised breakfast meal, resulted in reduced postprandial insulin concentrations in healthy adults, with no effects on glucose or GLP-1 levels (Study 1). We observed no effect of increasing doses of CPH on glucose, insulin or GLP-1 in healthy adults above 60 years (Study 2). A daily intake of 4 g of CPH for eight weeks, was not capable of affecting glucose or lipid profile, body composition, ghrelin, adipokines or inflammatory markers in overweight and obese participants with MetS (Study 3). The possible effects of CPH in subjects with metabolic abnormalities such as MetS should be further explored, due to the limited number of included participants.

List of Publications

Paper I

Dale H.F.*, Jensen C.*, Hausken T., Lied E., Hatlebakk J.G., Brønstad I., Hoff D.A.L., Lied G.A.:

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.

Paper II

Jensen C.*, Dale H.F.*, Hausken T., Lied E., Hatlebakk J.G., Brønstad I., Lied G.A., Hoff D.A.L.:

Supplementation with cod protein hydrolysate in older adults: a dose range cross-over study. *J Nutr Sci*. 2019;8: e40. Epub: 02/12/2019.

Paper III

Jensen C., Dale H.F., Hausken T., Hatlebakk J.G., Brønstad I., Lied G.A., Hoff D.A.L.:

Supplementation with Low Doses of a Cod Protein Hydrolysate on Glucose Regulation and Lipid Metabolism in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study. *Nutrients.* 2020, 12(7):1991. Epub: 09/07/2020.

Paper IV

Jensen C., Dale H.F., Hausken T., Hatlebakk J.G, Brønstad I., Lied G.A., Hoff D.A.L.

The Effect of Supplementation with Low Doses of a Cod Protein Hydrolysate on Satiety Hormones and Inflammatory Biomarkers in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study. *Nutrients.* 2020, 12(11):3421. Epub: 12/11/2020.

*Contributed equally

Disclaimer

Two of the clinical intervention studies included in the current PhD thesis were performed in collaboration with PhD candidate and colleague Hanna Fjeldheim Dale, employed on the same project. Papers I and II in this thesis are based on the work from these studies and holds shared authorship with Hanna Fjeldheim Dale. These two papers are also included in the PhD thesis of Hanna Fjeldheim Dale, defended April 2020, as they report major findings important for the overall understanding of the project.

Contents

1. INTRODUCTION

1.1 METABOLIC SYNDROME

Metabolic syndrome (MetS) is a cluster of metabolic disturbances, including hyperglycaemia, hypertension, dyslipidaemia and abdominal obesity (1-3). It has become a common metabolic disorder, resulting from the increasing proportion of overweight and obese individuals observed globally (3). The presences of MetS increases the risk of non-communicable diseases (NCDs), such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (4). The NCDs caused 71% of all deaths in 2016 and represents a major global health challenge, with cancer, chronic respiratory disease, diabetes and CVD responsible for the majority of these deaths (5, 6). The NCDs is a challenge for sustainable development (7), defined by the Brundtland Commission in 1987 as a development that "*meets the needs of the present without compromising the ability of future generations to meet their own needs"* (8). The reduction of the burden and mortality of the NCDs through prevention and treatment, are set as goals in both the World Health Organization (WHO) Global NCD Action Plan (9) and the United Nations' 17 Sustainable Development Goals (SDGs) (7).

1.1.1 Definition

MetS originates back to the 1920's, first described as the "hypertension-hyperglycaemia-hyperuricemia-syndrome" by the Swedish physician, Eskil Kylin (3, 10). In 1947, Vague observed that android obesity (upper body adiposity) was associated with abnormalities in the metabolism involved in the development of T2DM and CVD (11, 12). In the following years, several research groups described the clustering of different components of the MetS, and it was given different names such as the "metabolic trisyndrome" or the "plurimetabolic syndrome" (13). In 1988, the role of insulin resistance was emphasized by Reaven, who named it the "Syndrome X" (14). This was followed by Kaplan that added central obesity, and described the syndrome as the clustering of central obesity, hypertension, hypertriglyceridaemia and

impaired glucose tolerance, calling it “The Deadly Quartet” (15). In later years, it has also been referred to as the insulin resistance syndrome (3, 13).

Several different diagnostic criteria and definitions of MetS have been proposed over the years. The first attempt to create an internationally acknowledged consensus definition came in 1998, when WHO proposed a working definition for MetS (16). It included glucose intolerance, impaired glucose tolerance, diabetes mellitus and/or insulin resistance, with two or more of the following components: hypertension, increased levels of triacylglycerol (TAG), reduced levels of high-density lipoprotein cholesterol (HDL-C), central obesity or body mass index (BMI) >30 kg/m^2, or microalbuminuria (16). A modification was presented by the European group for the Study of Insulin Resistance in 1999, but with rather small changes from the proposal by WHO (17). The National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) released a new definition in 2001, where three out of five risk factors qualified for the diagnosis of MetS (abdominal obesity, elevated TAG, fasting glucose or blood pressure (BP), and reduced HDL-C (18). In 2005, The International Diabetes Federation (IDF) released a definition where abdominal obesity was made obligatory as one of the five factors required to qualify for MetS, with the remaining criteria identical to the definition by NCEP ATP III (1).

In 2009, the IDF; the American Heart Association; National Heart, Lung, and Blood Institute; International Atherosclerosis Society; World Heart Federation and International Association for the Study of Obesity, formed a harmonized, unified definition of MetS called the Joint Interim Statement (JIS) (1). In this definition, any three of five risk factors qualify for a diagnosis of MetS: increased waist circumference (WC), elevated BP, fasting glucose or TAG, or reduced level of HDL-C. The specific criteria and cut-off points are given in **Table 1.** For WC, it is recommended to use population- and country specific cut-off points, because of the complexity of defining a threshold for abdominal obesity similar in all ethnic groups and populations (1). Several different thresholds are proposed. The IDF suggests using the threshold ≥80 cm (women) and ≥94 cm (men) in European population (19). The WHO propose that the above-mentioned thresholds indicate an increased metabolic

risk in Caucasian populations, with WC ≥88 cm in women and ≥102 cm in men, indicating an even higher metabolic risk (1).

Table 1. Criteria and cut-off points for defining the Metabolic Syndrome according to the Joint Interim Statement definition.

Criteria	Cut-off points
Waist circumference	Population and country-specific
Fasting serum glucose*	≥5.5 mmol/L (100 mg/dL)
Serum TAG*	≥1.7 mmol/L (150 mg/dL)
Blood pressure*	SBP ≥130 mmHg and/or DBP ≥85 mmHg
Serum HDL-C*	Men: <1.0 mmol/L (40 mg/dL) Women: <1.3 mmol/L (50 mg/dL)

Adapted from Alberti et al. 2009 (1). TAG; triacylglycerol, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL-C: High-density lipoprotein cholesterol. Three of five risk factors qualify for a diagnosis of MetS. *Drug treatment for elevated TAG, fasting glucose or reduced HDL-C, or antihypertensive drugs are alternative indicators for MetS.

1.1.2 Prevalence

It is challenging to compare the prevalence of MetS across different studies (20). The prevalence estimates are affected by the definition used for MetS and the population studied, with factors such as gender, age, race, ethnicity, lifestyle and socioeconomic status affecting the estimates (20). A consistent finding across studies, is the increasing prevalence of MetS with increasing age (3).

The prevalence of MetS is relatively high globally, and it appears to increase with a parallel rise in prevalence of obesity (21). In a study comparing subjects from 12 different cohorts across 10 European countries and one from the United States, the overall prevalence of MetS was 24.3% (22). In Norway, similar numbers have been reported. The prevalence of MetS was investigated among participants in a Norwegian population study, the Nord-Trøndelag Health Study 1995-1997 (HUNT 2), by using the IDF-definition and 2005 ATP III criteria for MetS (23). For both definitions, the prevalence of MetS increased with increased age. A higher prevalence of MetS was reported in all demographics groups when using the IDF-definition compared to the 2005 ATP III criteria, with the exception of men aged 20-29 years. In total, 29.6% (95% CI: 28.8, 30.5) of the population had MetS when using the IDF-definition, compared to 25.9% (95% CI: 25.0, 26.7) with the 2005 ATP III criteria (23). This is

similar to estimates reported among participants (age 40-49 years) in the Hordaland Health Study (1997-1999): 30% had MetS according to the JIS-definition, with a higher prevalence among men (37.3%) than women (24.6%) (24). When using the same definition, data from the Tromsø 4 (1994-1996, age 26-70 years) and Tromsø 6 study (2006-2008, age 30-87 years), revealed that the prevalence of MetS was 8.1 % and 22.7 %, respectively, in these populations (25, 26).

1.1.3 Management

The primary goal in the management of MetS, is to reduce the risk of CVD and T2DM development (27). By targeting underlying, modifiable factors of MetS, such as obesity, physical activity, and diet, it is possible to improve metabolic components affected by the syndrome and thereby alleviating the risk of CVD and T2DM (3, 27). Lifestyle changes, combining weight reduction, increased physical activity and diet modifications, are the first-line therapy for MetS. As some individuals with MetS are at high or moderately high risk for major CVD events within the next 10 years (short term), additional drug therapy might be necessary to be able to reduce the CVD risk. If a given risk factor (e.g., BP or glucose levels) is severely abnormal, drug therapy may also be required. It is important to underline that irrespective of risk status and the need for drug therapy, lifestyle changes should be part of the management (3, 27).

The American Heart Association and the National Heart, Lung and Blood Institute recommends that weight reduction is achieved through behavioural change, with a combination of reduced caloric intake and increased physical activity (3, 27, 28). The initial goal is to achieve a weight loss of between 5-10% of initial body weight, by reducing weight by 0.5-1 kg per week, during a period of 6 to 12 months (27, 29). The metabolic risk factors of MetS, such as dyslipidaemia, hypertension, hyperglycaemia, will be reduced by achieving the recommended weight loss, thereby reducing the severity of the syndrome (27). These recommendations for weight reduction are in line with the Norwegian Directorate of Health's advice for individuals with obesity, and it is emphasized that patients with MetS may achieve significant health benefits by weight loss, such as reduced risk of T2DM (30). A recent meta-analysis observed significant improvements in metabolic risk factors and reduced prevalence of MetS,

after a supervised multifaceted lifestyle intervention, combining dietary intervention and physical activity (31). The combination of dietary intervention with physical activity seems to result in greater weight loss in the long-term, compared to a dietary intervention alone (32). Additionally, increased weight loss and beneficial effects on metabolic risk factors are observed with increased adherence (33, 34).

In general, it is important that the initial phase is followed by monitoring and long-term follow-up, with maintenance of weight loss, physical activity and diet change (3, 27, 30). Adhering to a dietary intervention and sustaining the weight loss over time are proven to be difficult (33, 35), and weight-regain is common (36). Furthermore, there is conflicting evidence regarding conventional strategies and long-term weight loss (37-39). Pharmacotherapy is often used adjunct to lifestyle change, increasing and maintaining the weight reduction (40). These therapies have great potential, but there is a need for development, due to moderate weight loss, weight regain after drug termination and side effects (41). Bariatric surgery is an alternative weight-loss strategy (41). Compared to conventional therapies, this method is effective in the short-term, resulting in a greater weight loss, with improved metabolic risk factors and comorbidities (42, 43). However, these procedures are associated with greater risk and possible side effects on short- and long-term (41, 44).

Considering the challenges of a achieving a successful long-term weight loss, and side effects of alternative strategies, exploring other strategies that may prevent the development of MetS is of interest. The intake of fish is suggested to play a protective role (45), and the consumption of fish is associated with reduced risk of MetS in cross-sectional studies (24-26). There is also evidence of beneficial changes in the specific components of MetS after the consumption of lean fish (46, 47). Despite a growing number of animal studies reporting beneficial effects on metabolic risk factors after supplementation with fish protein hydrolysates (48-52), there are few intervention studies in humans, with conflicting results (53-57).

To the best of our knowledge, a dietary supplement with a low dose of a fish protein hydrolysate has not been explored in overweight and obese humans with MetS, which is the primary focus of this thesis.

1.1.4 Pathophysiology

The development of MetS is complex, and not fully understood. Various pathophysiological explanations have been proposed, with insulin resistance as the most widely accepted hypothesis (3, 58). An overview of the proposed organ systems and mechanisms involved in the development of MetS is given in **Figure 1**. A sedentary lifestyle and excessive caloric intake over time, combined with a genetic predisposition, are suggested to be involved in the initiation of MetS (59). Nutrient surplus leads to increased storage of fat in adipocytes, including increased number and size. Over time, the adipocytes ability to store TAGs are exceeded or impaired, resulting in increased secretion of free fatty acids (FFAs) to circulation and lipid accumulation in the liver and skeletal muscle, causing insulin resistance (59).

In the skeletal muscle, overabundance of FFA inhibit the insulin-mediated glucose uptake and result in reduced insulin sensitivity in muscle tissue (3). The increased glucose levels in circulation, will lead to increased secretion of insulin from the pancreas, causing hyperinsulinaemia. This may further contribute to the development of hypertension, through enhancement of sodium reabsorption in the kidneys. In the liver, the overabundance of FFA increase the production of TAG and glucose, and the secretion of very-low-density lipoprotein cholesterol (VLDL-C), resulting in increased density of low-density lipoprotein cholesterol (LDL-C) and decreased levels HDL-C (3). A low-grade inflammation has also been implicated as part of the pathophysiology of the MetS, with increased secretion of pro-inflammatory cytokines from the adipose tissue (3, 58). This is further reviewed in subchapter 1.1.9.

In the next subchapters, the pathophysiology of MetS will be elaborated further by addressing the different components of syndrome in more detail.

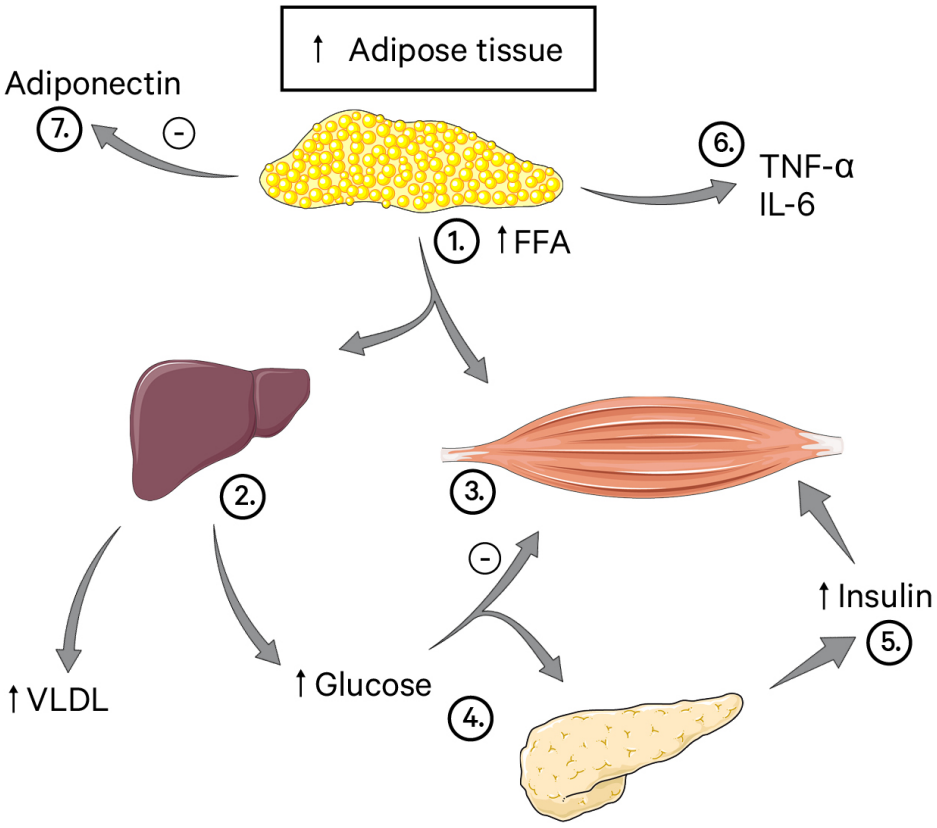

Figure 1. Pathophysiology of the metabolic syndrome. As a result of an increased and expanded adipose tissue, free fatty acids (FFAs) are released to circulation (1). In the liver, the FFAs causes increased production of glucose and TAG, including increased excretion of VLDL (2). Related lipoprotein-alterations are decreased levels of HDL-C, with increased density of LDL-C. In skeletal muscle, the increased FFAs causes insulin resistance (3), and inhibit the uptake of glucose. Increased levels of glucose in blood (4) will cause the pancreas to release more insulin (5), resulting in hyperinsulinaemia. At the same time, an inflammatory process in the adipose tissue, cause the secretion of inflammatory cytokines, such as IL-6 and TNF-α (6). The increased adipose tissue will also inhibit secretion of adiponectin (7). The figure is inspired by Eckel et al (2005) (3). The medical images are taken from Smart Servier Medical Art. The figure is made in Adobe Illustrator by Jensen 2020. FFA: Free fatty acid, TAG: triacylglycerol, VLDL: very-low density lipoprotein, IL-6: Interleukin-6, TNF-α: Tumor necrosis factor alpha.

1.1.5 Glucose metabolism

The MetS is characterized by alterations in glucose metabolism, with fasting glucose levels above 5.5 mmol/L considered one of the criteria for the diagnosis of MetS (1). In healthy individuals, fasting glucose levels are regulated within a narrow range of 4 to 6 mmol/L, with abnormal levels quickly regulated to maintain glucose homeostasis (60). In response to low glucose levels, glucagon is secreted from the α-cells of the pancreas, leading to the breakdown of glycogen in the liver and release of glucose, (61). This results in increased glucose levels (61). On the other hand, high glucose levels in circulation causes secretion of insulin from the pancreatic ß-cells (60). This results in increased uptake of glucose in the skeletal muscle and adipose tissue, returning the glucose levels to their normal fasting levels (60). Insulin works through binding to an insulin receptor at the plasma membrane of the cells, activating signalling pathways such as phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), resulting in the translocation of the glucose transport protein 4 (GLUT4) from storage site in the cells to the plasma membrane, enabling uptake of glucose into the cell (60, 62). The activation of PI3K is inhibited by reduced phosphorylation of tyrosine, which is linked to increased fat accumulation in peripheral tissue seen in MetS (58). There is also an inhibited activation of Akt, which is associated with increased levels of Acyl-CoA, known to be involved in metabolism of fatty acids (58). When target tissues, such as muscle and liver, are unable to respond to normal circulating concentration of insulin, insulin resistance has developed (63). This causes less glucose to be absorbed into the cell, resulting in elevated glucose concentration in blood. To compensate, the ß-cells secretes more insulin to maintain euglycaemia, causing hyperinsulinaemia. Over time, the ß-cells will not be able to maintain the high insulin production due to functional defects, leading to impaired glucose tolerance and eventually the development of T2DM (63). It is suggested that increased levels of FFA released from a saturated adipose tissue contribute to the development of insulin resistance seen in MetS (3, 58).

Observed effects on glucose metabolism after the intake of cod protein or fish protein hydrolysates

Several animal studies have observed beneficial effects on parameters of glucose metabolism after the intake of cod protein (64-67). These findings are partly confirmed in human intervention studies. Insulin-resistant individuals consuming a cod protein-containing diet had improved insulin sensitivity after 4 weeks, when compared to other animal proteins (68). Postprandially reduced insulin levels and higher glucose response were observed in an acute-effect study, after participants consumed a test meal with cod protein, compared to meals with milk or soy protein (69). Decreased postprandial levels of insulin-C peptide were observed in healthy adults consuming a diet with 60% of dietary protein from lean seafood (cod, pollock, saithe and scallop) for 4 weeks, compared to a non-seafood diet (70). No effect on fasting or postprandial levels of insulin or glucose were observed in the latter study (70), in line with studies reporting no effect on fasting or postprandial levels of glucose or insulin after a high intake of cod (750 g/week) in normal-weight (71) or overweight and obese (72) subjects. A daily consumption of 100 g lean fish per day for 8 weeks, did not significantly affect serum levels of glucose or insulin, or insulin resistance in individuals with MetS (47).

The large amounts of by-products occurring within the fisheries and aquaculture every year, are increasingly recognized as a high-quality source of protein (73). A promising approach for utilization is the production of fish protein hydrolysates, elaborated on in subchapter 1.2.4 (74). Protein hydrolysates made from salmon and herring are reported to have antidiabetic effects in animals (49). Reduced postprandial glucose level (49) and improved insulin sensitivity (51) have been observed in rats fed with diets containing salmon protein hydrolysate, with no effect on fasting glucose levels or glucose tolerance when rats were fed protein hydrolysates from herring, mackerel, bonito or salmon (51). A lack of effect on parameters of glucose metabolism was also observed in rats fed a diet with saithe protein hydrolysate, compared to casein- or soy-fed rats (75). Reduced non-fasting insulin and glucose in serum and the liver were observed in rats fed a high-fat diet containing salmon protein hydrolysate as the only

protein source (50). These changes, combined with a reduced expression of a specific gene, the PcK1-expression, are proposed to indicate better insulin sensitivity in the salmon-hydrolysate fed rats (50).

In humans, few studies have investigated the supplementation of low doses of fish protein hydrolysates on glucose metabolism (53, 54, 57), with inconclusive findings and various study populations. No effect on serum levels of insulin or glucose were observed after supplementation with 5.2 g of blue whiting hydrolysate for 6 weeks in 21 older adults living in a nursing home setting, when compared to placebo (53). In an acute-effect study in overweight and obese individuals, levels of glucose, insulin and insulin C-peptide were not significantly affected by a single dose of 3 g fish peptide hydrolysate made from bonito (54). In contrast, 2 g of blue whiting hydrolysate (1 g taken twice daily) supplemented to overweight subjects, resulted in reduced postprandial glucose levels after an *ad libitum* lunch meal compared to placebo, with no effect on insulin concentrations (57). Other studies have evaluated the effect of low doses of unhydrolysed cod protein in healthy normal weight, overweight and obese subjects, with varying results. A daily supplement of cod protein (3 or 6 g per day) for 8 weeks, had beneficial effects on postprandial glucose and reduced fasting glucose levels in overweight and obese subjects, with no effects on insulin or insulin C-peptide (76). In line with these results, reduced postprandial glucose were observed after 8 weeks supplementation with 2.5 g of cod protein in overweight and obese individuals (only within-group difference), with no effect on insulin, insulin C-peptide or fasting glucose levels (77). A daily intake of a higher dose of cod protein (6 g/day), with the same intervention length, did not significantly affect fasting glucose or insulin levels in overweight and obese subjects, when compared to placebo (78). Similarly, 8 weeks supplementation with 8.1 g/day of cod protein in healthy, normal weight adults, had no effect on fasting serum concentration of glucose and insulin, or postprandial concentration of insulin, with postprandial glucose concentrations only decreased at one time point (60 minutes), when compared to control (79). They did however observe decreased concentrations of α-hydroxybutyrate, β-hydroxybutyrate and acetoacetate compared to the control group, suggested to reflect early changes in regulation of glucose (79).

1.1.6 Dyslipidaemia

MetS is characterised by dyslipidaemia, with elevated levels of TAG and reduced levels of the lipoprotein particle HDL-C, including increased levels of VLDL-C and LDL-C (80). As TAG and cholesterol are lipophilic molecules, they must be transported in blood in the form of lipoproteins (81). Lipoproteins are water-soluble macromolecules, composed of an inner, hydrophobic core of TAG and cholesteryl ester, with an outer membrane made up of apolipoproteins, phospholipids and unesterified cholesterol. Based on density, the lipoproteins can be classified into different subclasses: chylomicrons, VLDL-C, intermediate density lipoprotein, LDL-C and HDL-C (81). Each class of lipoprotein contain a specific combination of apolipoprotein, important for their function and metabolism of lipoproteins (82). The chylomicrons are formed in the gastrointestinal (GI) tract, transporting TAG and cholesterol derived from diet from the intestine to various cells (82). As the concentration of TAG are reduced in the chylomicrons, cholesterol-rich particles called chylomicron remnants are formed. VLDL-C is formed in the liver from the chylomicron remnants, transporting TAG and cholesterol to peripheral tissue. Through hydrolysis of TAG by lipoprotein lipase, the VLDL particle is depleted of TAGs, leading to the formation of smaller cholesterol-enriched particles (82). LDL-C, a breakdown product of VLDL-C, is rich in cholesteryl esters and delivers cholesterol to peripheral tissue and liver (83). Through the reverse cholesterol transport, excess cholesterol from peripheral tissues is removed and transported to the liver for uptake by HDL-C (81).

During development of MetS, ectopic fat accumulation and overabundance of FFAs released to circulation (59), causes increased hepatic production and secretion of VLDL-C to the bloodstream (3). Additionally, insulin resistance may cause reduction in peripheral tissue levels of lipoprotein lipase (3), an enzyme degrading TAG to fatty acids and glycerol (82). These events contributes to the development of elevated TAG, or hypertriglyceridemia, a common metabolic disturbance seen in individuals with MetS (3). Elevated levels of TAG above 1.7 mmol/L is one of the criteria for MetS when using the JIS-definition (1). It has been observed that when elevated fasting

TAG levels are above 2.0 mmol/L, most individuals will exhibit changes in the composition of LDL-C, having more of atherogenic small, dense LDL-particles (3). Reduced levels of HDL-C is another criterion for MetS in the JIS-definition (1). The homeostasis of cholesterol is maintained through the interactions of de novo biosynthesis, uptake, export and storage of cholesterol (84). Cholesterol is a vital molecule, produced in the body or derived from the diet. The major site of cholesterol synthesis in the body is the liver (84). A reduced cholesterol content of HDL-C is observed in the presence of hypertriglyderdaemia (3). This is caused by changes in the lipoprotein core of HDL-C, with reduced levels of cholesteryl ester and increased levels of TAGs, resulting in a small, dense lipoprotein particle. The changes in composition and metabolism of the lipoprotein particles play a role in the reduced levels of HDL-C observed in some patients with MetS (3).

Observed effects on lipid metabolism after intake of cod protein or fish protein hydrolysates

In cross-sectional studies, increased consumption of lean fish has been associated with decreased levels of TAG and increased levels of HDL-C (24, 25, 46). In animal studies, beneficial effects on cholesterol (85-87) and TAG (65) have been observed after the intake of cod protein, with no significant effects on lipid markers in other studies (64). Conflicting results are also reported from human intervention studies. Reduced fasting and postprandial serum concentrations of TAG, with prevention of VLDL particle concentration elevation, were observed in healthy subjects consuming a lean seafood diet for 4 weeks, compared to a non-seafood diet (88). No significant effects on fasting or postprandial levels of total cholesterol (total C), LDL-C or HDL-C were observed (88). Decreased levels of TAG have also been reported after daily consumption of cod (150g/day) for two weeks in healthy subjects (89), whereas no significant effects on lipid markers were observed after a high intake of cod (750 g/week) in normal weight subjects after 4 weeks (71) or in overweight subjects after 8 weeks (90). In individuals with MetS, a daily consumption of 100 g lean fish for 8 weeks significantly reduced LDL-C levels, with no significant effect on other lipid markers (47).

Bioactive peptide sequences with potential cholesterol-lowering effects have been observed in protein hydrolysates made from salmon and herring (49). Decreased concentration of HDL-C was observed in rats fed a diet with herring protein hydrolysate (49) or salmon protein hydrolysate (52), with no effect observed in rats fed a diet with saithe protein hydrolysate (75). In rats fed a high-fat diet containing salmon protein hydrolysate, reduced levels of postprandial TAG in both serum and liver were observed (50), with lower fasting levels of plasma TAG reported in rats fed a diet with saithe protein hydrolysate (75). Other animal studies report of no significant effect on fasting levels of TAG after supplementation with fish protein hydrolysates (48, 49, 51), with increased levels reported in some (49). Conflicting results are also observed for total C, with reduced levels reported in rats fed a salmon protein hydrolysate containing-diet (52), with no significant effects in mice fed a diet with salmon protein hydrolysate (48) or rats fed saithe protein hydrolysate-containing diet (75).

An acute-effect study observed no significant effects on postprandial levels of TAG in overweight and obese subjects, after a single, low dose of fish protein hydrolysate (3 g, made from bonito), when compared to placebo, a vitamin D-supplementation or a combined intervention of both (54). To our knowledge, no other study has evaluated the effect of a low dose of a fish protein hydrolysate on lipid metabolism in a human intervention study. Other studies have investigated the effect of low doses of non-hydrolysed cod protein (intact), with a lack of significant findings (76, 77, 91).

1.1.7 Abdominal obesity

Overweight and obesity are defined as "*abnormal or excessive fat accumulation that may impair health*" (92). Obesity is a risk factor for the development of NCDs (93), and associated with increased risk of CVD and T2DM (92, 94-96), musculoskeletal disorders (97) and certain cancer forms (98). The prevalence of obesity is increasing across all regions of the world (99, 100), with obesity recognised as a significant driving force behind MetS (3). According to the latest report from the WHO, 39% of adults were overweight and about 13% were obese in 2016 (92). BMI is a simple and widely used index to classify overweight and obesity in adults, and is defined as "*a person's weight in kilograms divided by the square of his height in meters (kg/m^2)*". Overweight is defined as a BMI between 25-29.9 kg/m^2, whereas obesity is defined as BMI ≥30 kg/m^2 (92). A BMI ≥30 kg/m^2 is observed among the majority of individuals with MetS, and those with abdominal obesity seems to be of higher risk for MetS (80). Clinically, abdominal obesity is presented as increased WC (101). Abdominal obesity is associated with the metabolic risk factors, and the different products secreted from an expanded adipose tissue, such as cytokines and nonesterfied fatty acids, seems to worsen these risk factors (101).

Observed effects on abdominal obesity after the intake of cod protein or fish protein hydrolysates

Inconclusive findings are reported on the consumption of lean fish in humans and effects on WC (25, 26, 46, 47, 102, 103). In animal studies, rats fed a high-fat diet containing salmon protein hydrolysate had lower body weight gain and less visceral adipose tissue (51), with reduced abdominal tissue mass observed in rats fed a diet with saithe protein hydrolysate (75). A dietary supplement with 1.4 g or 2.8 g of fish protein hydrolysate taken daily for 3 months in overweight subjects combined with a caloric deficit of 300 kcal, had a beneficial effect on abdominal obesity in overweight subjects, shown as reduced WC, with reduced body weight, fat mass and BMI (56). No effect on body weight, percentage body fat or muscle were observed after a daily intake of a low dose of cod protein (3-6 g, intact) for 8 weeks in overweight subjects, without caloric restrictions (76).

1.1.8 Arterial hypertension

An elevated blood pressure, defined by the JIS-definition as SBP ≥130 mmHg and/or DPB ≥85 mmHg or the use of antihypertensive medications, is considered another component of the MetS (1). It has been suggested that insulin resistance, seen as part of the MetS, is involved in the development of hypertension by increasing the reabsorption of sodium in the kidneys (3). Other mechanism proposed in combination with this, is activation of the sympathetic nervous system and renin-angiotensin-aldosterone system, increased secretion of angiotensin from adipose tissue and an expanded intravascular volume (80).

Observed effects on blood pressure after the intake of cod protein or fish protein hydrolysates

The development of high blood pressure was prevented in rats fed cod protein (104), with conflicting results in human intervention studies. A lower blood pressure was observed after the consumption of lean fish in patients with coronary heart disease (105) and with MetS (47), whereas others report of no significant effects (106) or even increased blood pressure (102). Peptide sequences with known inhibitory effects on the angiotensin-1 converting enzyme (ACE) have been identified in salmon and herring protein hydrolysates (107). High ACE inhibitor activity has also been demonstrated in other fish protein hydrolysates (108), but when the different hydrolysates (cod, salmon, haddock) were fed to spontaneously hypertensive rats for 28 days, the expected increase in blood pressure was not inhibited (108). A decreased blood pressure and increased levels of plasma angiotensin 1, was observed in 29 individuals with mild hypertension after daily supplementation with fish protein hydrolysate for 4 weeks (55). The fish protein hydrolysate was made from sardine muscle and suggested to contain the bioactive sequence valyl-tyrosine (55). Another study found no significant effect on vascular function in healthy adults after a single low dose of 5 g of fish protein hydrolysate (109). As these results are reported from healthy adults, potential beneficial effects in individuals with hypertension or other pathological conditions need to be further explored before any conclusion can be drawn (109).

1.1.9 Low-grade inflammation

A low-grade chronic inflammation has also been implicated as part of the pathophysiology of MetS (58). Inflammation is the immune system's response to a harmful stimulus, protecting the host against infection and other insults, triggered by tissue damage, microbial products, or metabolic stress (110). The process activates several signaling pathways and releases inflammatory cytokines, resulting in an inflammatory response. This response is characterized by symptoms such as swelling, redness, pain, heat, and loss of function at the affected site. As soon as the trigger is eliminated, the inflammation is terminated through the release of anti-inflammatory cytokines and inhibition of proinflammatory signaling cascades, restoring the homeostasis of the inflamed area. The acute inflammation is essential for the host, but if not terminated properly it may evolve into a chronic inflammation. This is identified by change in cell types, with simultaneous destruction and healing of the affected tissue. Prolonged tissue damaged may occur, leading to development of disease, e.g., inflammatory bowel disease, with elevated levels of inflammatory biomarkers in affected tissue and systemic circulation, a so-called high-grade inflammation. Chronic inflammation can also be low-grade, observed in MetS and obesity, which is often asymptomatic, with a less pronounced increase in inflammatory biomarkers (110). As a response to the nutrient overload, the adipose tissue becomes infiltrated by macrophages, T cells and other immune cells, leading to an inflammatory response and secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 (58). TNF-α is produced mainly by lymphocytes and macrophages, with some in adipose tissue (111). IL-6 is produced by adipose tissue and several other cell types, and one of its main tasks is to stimulate production of C-reactive protein (CRP) in the liver (111). The increased levels of these circulating cytokines may contribute to development of insulin resistance in liver, muscle, and other tissue, as well as causing increased FFAs in circulation due to degradation of TAGs (3, 59). Increased levels of IL-6 have been observed in individuals with obesity and T2DM, and in individuals with MetS (58).

Observed effects on inflammatory markers after the intake of cod protein or fish protein hydrolysates

There are indications that intake of fish is linked to reduced inflammatory activity (112). Results from studies exploring the effect of cod fillet consumption on inflammatory markers are inconclusive, with beneficial effects reported in some studies (113, 114), with others reporting no significant effects (72). Animal studies have indicated that fish protein hydrolysates as part of the diet, may have anti-inflammatory effects. Reduced levels of TNF-α and IL-6 were observed in rats fed a high-fat diet containing protein hydrolysates from herring, mackerel, salmon or bonito for 28 days, compared to control fed rats (casein) (51). In a mouse model of chronic inflammation, the plasma levels of interferon gamma were reduced after a diet with salmon protein hydrolysate compared to control, but with no effects on other cytokines (IL-1 β, IL-2, IL-5 or GM-CSF) (48). In humans, two studies have evaluated the effect of low doses of fish protein hydrolysate on markers of inflammation (53, 115). Reduced levels of monocyte chemoattractant 1 (MCP-1) and increased serum concentration of CRP were observed in 21 nursing home residents after a daily supplement containing 5.2 g of a fish protein hydrolysate for 6 weeks, compared to placebo (53). In a study by our research group (115), a daily intake of 2.5 g fish protein hydrolysate made from cod for the same intervention length, did not affect pro-inflammatory cytokines (IL-8) or any of the gut integrity markers in patients with irritable bowel syndrome, compared to placebo (115).

1.1.10 Other manifestations of the metabolic syndrome

Adiponectin and leptin

Altered levels of adiponectin are suggested to be involved in the development of the MetS (3). Adiponectin is protein of 30 kDa, discovered in the 1990's (116-119), mainly produced and secreted from adipose tissue (111, 120). The adipose tissue has traditionally been viewed as a passive organ serving as a pool for energy, but is now recognised as an active endocrine organ involved in several physiological processes (111, 121). In healthy humans, adiponectin is found in high concentrations in circulation (120), with plasma levels reported to range between 5-30 μg/mL (111). Reduced levels of adiponectin have been observed in subjects with obesity and patients with T2DM (111, 122), including subjects with MetS (121). The levels of adiponectin seems to decrease with increased number of components of MetS (123), with decreased levels of adiponectin linked to deterioration of the metabolic risk factors (101).

Adiponectin is known to enhance insulin sensitivity (124, 125), by acting through two receptors expressed in muscle and liver: AdipoR1 and AdipoR2 (111). In the skeletal muscle, adiponectin binds to its receptor and activates adenosine mono phosphate activated protein kinase, promoting glucose uptake in muscle cells through translocation of GLUT4 to the cell membrane (111, 121). In the liver, adiponectin decreases gluconeogenesis by inhibiting the hepatic enzymes phosphoenolpyruvate carboxylase and glucose-6-phospatase, and stimulating fatty acid oxidation (111, 121). Furthermore, insulin sensitivity is enhanced through increased expression of an insulin receptor substrate protein in the liver, and by acting as an agonist in the peroxisome proliferator activated receptor gamma, leading to increased glucose uptake (121). Adiponectin also appear to have anti-atherosclerotic and anti-inflammatory properties (111). The activation and secretion of the pro-inflammatory cytokines in response to the infiltration of the oversaturated adipose tissue, may lead to reduced expression and secretion of adiponectin, through inhibition by IL-6 and TNF-a (126). The decreased

levels of adiponectin, combined with increased levels of pro-inflammatory cytokines, are suggested to be involved in the development of insulin resistance seen in MetS (3).

Furthermore, changes in leptin levels appear to be involved in the MetS (3). Leptin was identified as a protein encoded by the obese *ob* gene, with mutations in the gene causing obesity and T2DM in mice, resembling morbid obesity in humans (127). Leptin is produced and released by adipose tissue, and involved in regulation of appetite and body weight (128). The hormone mediates its effects by acting on regions in the hypothalamus involved in the control and regulation of appetite (129). The levels of leptin increase with increased fat mass, leading to suppression of appetite and a lower energy intake. When fat mass is reduced, the levels of leptin decline, resulting in appetite stimulation and increased energy intake. The levels are highly correlated with adipose tissue mass, and most obese subjects have high circulating levels of leptin. However, absence of an effect on appetite by leptin in obese subjects, suggest that they have developed leptin resistance (128, 129). Leptin resistance is proposed as an alternative pathophysiological explanation of MetS (3). It has also been suggested that the elevated levels of leptin seen in obese individuals contribute to low-grade chronic inflammation (130), involved in the progression of MetS (58).

There are indications that lifestyle modifications may influence adiponectin levels. Lifestyle intervention combining physical activity and energy-restricted diets, are shown to increase circulating levels of adiponectin, in the presence of weight loss (131). There are also observations that a daily intake of fish or long-chain n-3 polyunsaturated fatty acids (PUFAs) supplements may increase adiponectin levels (131). A fish protein hydrolysate fed to mice had no significant effect on fasting levels of adiponectin (48). In humans, increased levels of adiponectin (within-group) were observed after 8 weeks daily intake of 2.5 g cod protein in overweight and obese individuals (77). Reduced levels of leptin have been observed after lifestyle interventions (132, 133), with the effect of lean fish consumption on leptin levels being more conflicting (77, 102).

Appetite hormones

In recent years, there has been an improved understanding of how appetite regulation affects food intake, with more insight into how the gut hormones interacts with the central nervous system (134). The GI tract is considered the largest endocrine organ in the human body, producing and secreting several different GI hormones from the enteroendocrine cells, involved in the digestion, glucose homeostasis and energy balance (135). An overview of some of these GI hormones, their site of production and secretion, as well as main functions are given in **Table 2**. The GI hormones glucagon-like peptide 1 (GLP-1) and ghrelin will be elaborated in further detail in the next paragraphs, as these are relevant for the current thesis.

Table 2. Overview of important gastrointestinal hormones, their site of release and main function.

Hormone	Release site	Main function
Ghrelin	D/P1-cells, stomach	Hunger regulation
GLP-1	L-cells in small intestine and colon	Prolongs gastric emptying
		Stimulate insulin secretion
GIP	K-cells, small intestine	Stimulate insulin secretion
CKK	I-cells in duodenal and jejunal mucosa	Inhibit food intake
		Slows gastric emptying
PYY	L-cells in colon	Reduce appetite
INSL5	L-cells in colon	Promote appetite during starvation

GLP-1: Glucagon-like peptide 1, GIP: Glucose-dependent insulinotropic polypeptide, CCK; Cholecystokinin, PYY: Peptide-tyrosine-tyrosine, INSL5: Insulin-like peptide 5

Glucose-dependent insulinotropic polypeptide was the first identified incretin, followed by the identification of GLP-1 (136), which are defined as "*hormones that are secreted from the gastrointestinal tract into circulation in response to nutrient ingestion that enhance glucose-stimulated insulin secretion*" (136). GLP-1 are secreted from L-cells in the distal ileum and colon, rapidly secreted in response to nutrient ingestion, promoting the glucose-stimulated secretion of insulin. The hormone increase the secretion of insulin, by binding to a specific receptor on the pancreatic ß-cells, leading to the activation of adenylate cyclase activity, production of c adenosine monophosphate and stimulating several mechanisms. GLP-1 also increase the ß-cell

mass in the pancreas, through stimulating ß-cell proliferation and inhibition of apoptosis. Glucagon secretion and hepatic glucose production are inhibited by GLP-1, whereas the uptake and storage of glucose in muscle and adipose tissue are increased by the peptide hormone. GLP-1 inhibit gastric emptying, slowing down the transfer of nutrients from the stomach to the small intestine and thereby reducing the postprandial glucose levels. Beyond this, GLP-1 is known to promote satiety (136).

In humans, circulating GLP-1 is found mainly as GLP-1(7-36) NH_2 (136). The fasting levels of GLP-1 in healthy adults range between 5-10 pmol/L, increasing to 15-50 pmol/L after a meal (137). The secretion of GLP-1 occur in a biphasic pattern, with an early phase of GLP-1 secretion within the first 10-15 minutes after a meal, followed by longer second phase with GLP-1 secretion after 30-60 minutes. It is especially meals rich in carbohydrates and fats that stimulate the release of GLP-1 (136). GLP-1 have short half-life in circulation, and are degraded within minutes through cleaving by the proteolytic enzyme dipeptidyl peptidase-4 (DPP-4) (136, 138). By inhibiting DPP-4, the incretin hormone remains in circulation for a longer time, resulting in reduced glucose levels. This have led to the development of DPP-4 inhibitors, reducing the activity of DPP-4 by up to 80% and currently used as therapeutics in T2DM (137). In hydrolysates from fish, there have been identified bioactive peptides that have a similar DPP-4 inhibitory activity (139).

In contrast to GLP-1, that increase secretion of insulin, slows down gastric emptying and decrease appetite (136), the GI hormone ghrelin seems to have the opposite effects by inhibiting insulin secretion, accelerating gastric emptying and increasing appetite (140). Ghrelin was discovered in 1999, as a ligand to the growth hormone secretagogue receptor, stimulating the release of growth hormone (141). Subsequent studies reported that the peptide hormone also had effects on appetite and energy balance, by increasing food intake and body weight (142-144). Other functions have been described, such as involvement in gut motility and secretion of gastric acid, modulation of sleep and glucose regulation (140). Ghrelin is a 28 amino acid peptide hormone, originating from the cleaving of the precursor molecule preproghrelin (145), produced and secreted from enteroendocrine cells mainly located in the stomach, the

small intestine and pancreas (146). In circulation, two different forms can be detected: deacylated and acylated ghrelin. The latter is the biologically active, formed through acylation by the ghrelin O-acyltransferase, needed to activate the ghrelin receptor, GHS-R1a (140). The concentrations of ghrelin increase before a meal, followed by declining levels postprandially. After about one hour, ghrelin reach the lowest level, before it gradually increases until the next meal (147, 148). This profile fits well with the idea that ghrelin has a physiological role as a "meal initiator" or a "hunger hormone" (147). Despite large interindividual variations in ghrelin levels, reduced levels have been observed in overweight and obese subjects, compared to lean subjects (149, 150). There is currently no clear reference range for ghrelin, and the reported concentrations of ghrelin vary in the literature (151).

A few studies have reported on the effect of low doses of fish protein hydrolysates on circulating appetite hormones (56, 57, 152). Supplementation with 1.4 g or 2.8 g of fish protein hydrolysate made from blue whiting muscle for 90 days in overweight subjects, combined with a caloric deficit of -300 kcal, resulted in increased serum concentrations of GLP-1 and cholecystokinin, compared to placebo (whey protein isolate) (56). No statistically significant differences were observed when comparing the two different doses of fish protein (56). In contrast, a daily supplementation of 6 g cod protein (intact) for 8 weeks in overweight and obese individuals had no effect on GLP-1 levels (78). In line with these findings, no effect on GLP-1 or cholecystokinin, levels were reported after 2-week supplementation with 2 g of blue whiting hydrolysate in overweight subjects (57). The latter study did observe reduced sweet craving (self-reported sensation), with no effect on energy intake at an *ad libitum* lunch meal or subjective sensations of hunger (57). In an acute-effect study by our research group (152), a single weight-adjusted dose of fish protein hydrolysate made from cod had no effect on sensation related to feeling of hunger, serum fasting or postprandial levels of ghrelin in healthy subjects after a test meal (152). The last literature search for publications related to health effect of low doses of cod protein and fish protein hydrolysates in animal and human trials relevant for this thesis was done on December 28th, 2020.

1.2 FISH PROTEIN HYDROLYSATES

1.2.1 The importance of marine harvest in nutrition and health

The global population is estimated to increase from the current 7.7 billion to 9.7 billion by 2050 (153), increasing the demand for food and pressure on already strained natural resources (154, 155). A major global challenge is to transform our global food system and ensure food security for the growing population, with a need for innovative, sustainable solutions and better utilization of already harvested resources (155). According to the United Nations, food security is achieved when "*all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life*" (156).

Fisheries and aquaculture plays an important part in global food security through providing nutrition and employment, and will be crucial for securing food for future generations (73). Almost 60 million people rely on fisheries and aquaculture for employment, and 17% of the global intake of animal protein came from fish in 2017 (73). During the last 30 years the majority of growth within this sector has been from aquaculture, with 46% of the total global production of 179 million tonnes in 2018. Increased production within aquaculture is possible, and will be important for future global food security. To achieve this, a sustainable development is of great significance. It has been estimated that 35% of the global marine harvest is lost or wasted each year, including large amounts of valuable by-products being wasted (73). The sector have an important role towards the achievement of the SDGs by 2030, in particular SDG 14 "*Conserve and sustainably use the oceans, seas and marine resources for sustainable development*" and SDG 12 "*Ensure sustainable consumption and production patterns*" (7).

1.2.2 Dietary recommendations and nutrients in fish

Fish is an important part of the Norwegian diet, and we have long traditions of eating fish (157) and are one of the countries in Europe with the highest consumption (158). The average consumption of fish and fish products were estimated to be 67 g per day among Norwegian adults in Norkost 3 (2010-2011) (159). Men consumed more fish than women, with a higher consumption with increasing age (159). Despite a high intake of fish in Norway compared to other European countries, the consumption of fish has decreased since 2015, and only 30% of Norwegian women and 40% of Norwegian men reach the recommended intake of fish (160).

The dietary advice from the Norwegian Directorate of Health, recommending the general population to consume between 300-450 g of fish per week, is largely based on beneficial cardiovascular effects observed with an increased intake of the long-chain n-3 PUFAs (161). Overall, the observed health benefits of fish have to a large extent been attributed to the content of these fatty acids (162). This interest originates from studies published in the 1970s, proposing that the low incidence of ischemic heart disease and diabetes observed in Greenlandic Inuits were linked to their lipid profile (163). The beneficial lipid profile was suggested to be due the Inuit's lifestyle and a diet consisting of mainly fish and seal, with a high intake of the long-chain n-3 PUFAs (163, 164). The favourable association between fish consumption and CVD have been confirmed in later studies (165-169), while the association between fish and T2DM is less consistent (170-177).

The fatty and medium fatty fish species, such as Atlantic salmon, are considered the main sources of the long-chain n-3 PUFAs (161). When comparing different fish species, the fat content varies and fish can be classified as lean (<2% fat), medium fatty (2-5% fat) and fatty fish (>5% fat) (157). The lean fish species, such as Atlantic cod, contain less of the long-chain n-3 PUFAs, but are rich sources of iodine (161). Other important nutrients in fish are vitamin D and B12, selenium and protein of high-quality (178). In the studies by Dyerberg and Bang, the Greenlandic Inuits had a higher protein intake compared to Danish controls (164), which might suggest that protein content of fish was of importance for the observed beneficial lipid profile. In

recent years, several studies have observed beneficial health effects of lean fish consumption and proposed that the protein content may contribute to the health benefits of eating fish (179). Compared to the fat content, the protein content is relatively similar when comparing different fish species, and a fish fillet contains 15-20 g protein per 100 g fillet (wet weight basis) (178).

Fish have a lower carbon footprint compared to meat, and according to the report by "Nasjonalt Råd for Ernæring", reviewing the Norwegian dietary guidelines in a sustainability perspective, fish is sustainable to eat given that it comes from a sustainable fish stock or sustainable aquaculture industry (180). Despite fish considered being beneficial for health, it may also be a source of contaminants and undesired substances with potential hazard and negative effects, such as dioxins, dioxin-like polychlorinated biphenyls and mercury (178). The risk-benefit assessment conducted by the Norwegian Scientific Committee for Food and Environment in 2014, concludes that "*the benefits clearly outweighs the negligible risk presented by current levels of contaminants and other known undesirable substance in fish"* (178). An updated risk-benefit assessment is under preparation based on new available data, expected to be completed during the spring of 2021 (181).

1.2.3 Residual raw material

Large quantities of residual raw material remains when fish are gutted and further processed (182, 183), including heads, tongues, trimmings (cut offs), skin and bone, stomach and intestines (183). Residual raw material can be defined as "*the parts that are not the primary product when using the raw material*" (74). They can be classified in different groups based on origin and further processing, and if handled according to hygiene regulation they can be eaten or sold for animal-feed (183). Correct handling is critical for further processing, as fish is a perishable food item (73).

These residual raw materials have traditionally been discarded, used as feed for livestock, aquaculture, pets or animals, or in silage and fertilizer, which are low-value products, resulting in low turnover for the fishing industry (73, 184). In 2019, 965 000 tonnes of residual raw material occurred in Norwegian fisheries and aquaculture (74).

The utilization rate of these materials has continuously increased in the last couple of years, and the estimated utilization rate of 84% in 2019 is the highest reported (74). There is still large amounts of residual raw materials that are discarded every year, contributing to a significant loss of value of already harvested resources (74, 183). In Norway, it has been mainly residual raw materials from the white fish sector that have not been fully utilized, due to lack of space and technological solutions on the fishing fleets and economic incentives to bring the residual raw material to land (74). Of 155 000 tonnes unutilized residual raw material in 2019, 70% was estimated to come from the whitefish sector (74). Despite a positive development within this sector, almost 40% of the residual material from white fish is still discarded each year (74, 183).

In the Norwegian fishing and aquaculture industry, the residual raw materials that are further utilized are processed into various products (74). The majority of the residual raw materials are processed to silage, ending up as fish and livestock feed, biogas and energy. It is estimated that around 70% of residual raw materials end up as various components in feed, with 10% processed to products for human consumption. Around 3% is used indirectly for human consumption, through further processing to cod liver oil or protein extracts. In recent years, there has been a growing interest in the processing of residual raw material to fish protein concentrates and fish protein hydrolysates, with an overall increase of 21% from 2018 to 2019 (74). Increased utilization of the residual raw material will contribute to increased profits and reduced waste for the industry, contributing to more sustainable fisheries and aquaculture. This will also play an important part in the work towards achieving the SDGs within 2030, in particular SDGs 12 and 14, and if the residual raw materials can be used for improved health in humans, it will be of importance for SDG 3 *"Ensure healthy lives and promote well-being for all at all ages"*(7).

1.2.4 Production of fish protein hydrolysates

The residual raw materials from fish are increasingly recognised as a valuable source of nutrition that may be transformed into high-value products used for human consumption with potential beneficial health effects (73, 184, 185). The production of water-soluble fish protein hydrolysates is a promising approach to exploit these marine residual materials. A protein hydrolysate can be made by chemically or enzymatically degradation of protein to small chains of peptides of various size, by cleaving the bonds between amino acids (183). Chemical hydrolysis, including acid and alkaline hydrolysis, is an inexpensive and simple method (185). However, it is difficult to control the process with unspecific cleaving of peptide bonds, resulting in a heterogenous end product (185).

Enzymatic hydrolysis, on the other hand, allows for a more controlled process, resulting in a high-value end product with good functional, biological and nutritional properties (185). The hydrolysate is made by adding commercially available enzymes, with predefined conditions of pH, temperature and time, to the residual raw material (182, 184, 185). Through the hydrolysis process the raw material is degraded, resulting in a lipid layer at the top, an aqueous phase in the middle and a mixture of solids and non-soluble proteins at the bottom (185). The peptide-containing soluble fraction can be collected by separating it from the lipid layer, followed by spray- or freeze-drying and storage until use (185). The end product will vary in terms of bioactivity, bitterness and taste, depending on the choice of enzyme, type of raw material used and hydrolysis condition (184). The protein hydrolysates usually contain between 2-20 amino acids, and both the size and molecular weight of the peptides will affect the properties of the hydrolysate (185).

1.2.5 Digestion and absorption of protein and protein hydrolysates

Proteins are made up by long chains of amino acids connected by peptide bonds forming polypeptide chains, folded into three-dimensional structures (186). The structure and function of the protein is determined by the sequences of amino acids (186). Proteins have several important functions in the human body, being essential for

growth and maintenance of the human body, incorporated in several hormones and enzymes, precursors for neurotransmitters, and serving as a source of energy and amino acids (187). The amino acids can be classified as non-essential and essential, depending on the human body's ability to synthesize these amino acids. Whereas the majority can be synthesized from other amino acids within the body (non-essential), the essential amino acids must be provided through the diet. The essential amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and histidine (187). The protein in fish is considered to be of high-quality, due to a balanced amino acid profile with a high content of essential amino acids (178). The low amount of connective tissue and a high bioavailability makes fish easily digested (178).

The digestion of dietary protein begins in the stomach, by unfolding and cleaving of proteins into polypeptides by the enzyme pepsin (188). In the small intestine, proteases secreted from the pancreas and proteolytic peptides located on the brush border of the enterocytes, continue the digestion process through further hydrolysis of the proteins to di- and tripeptides and free amino acids (188). Free amino acids are absorbed through amino acid transporters, whereas the di- and tripeptides are absorbed through the peptide transporter, Peptide transporter 1 (PepT1), expressed in abundance in the small intestine (189). Some peptides (low-molecular) may also be transported by passive diffusion through water channels between the cells (190). A general overview of the digestion and absorption of proteins is shown in **Figure 2.**

A protein hydrolysate has been shown to increase digestion and absorption, resulting in a higher availability in plasma, as compared to its intact protein (191). It may therefore by hypothesized that a protein hydrolysate may affect the secretion of GLP-1, GIP and insulin, resulting in a more efficient uptake of glucose into the cells. However, the evidence is unclear, and most studies have investigated the effect on casein or whey proteins (191, 192), and it is unclear if fish protein elicits the same effects.

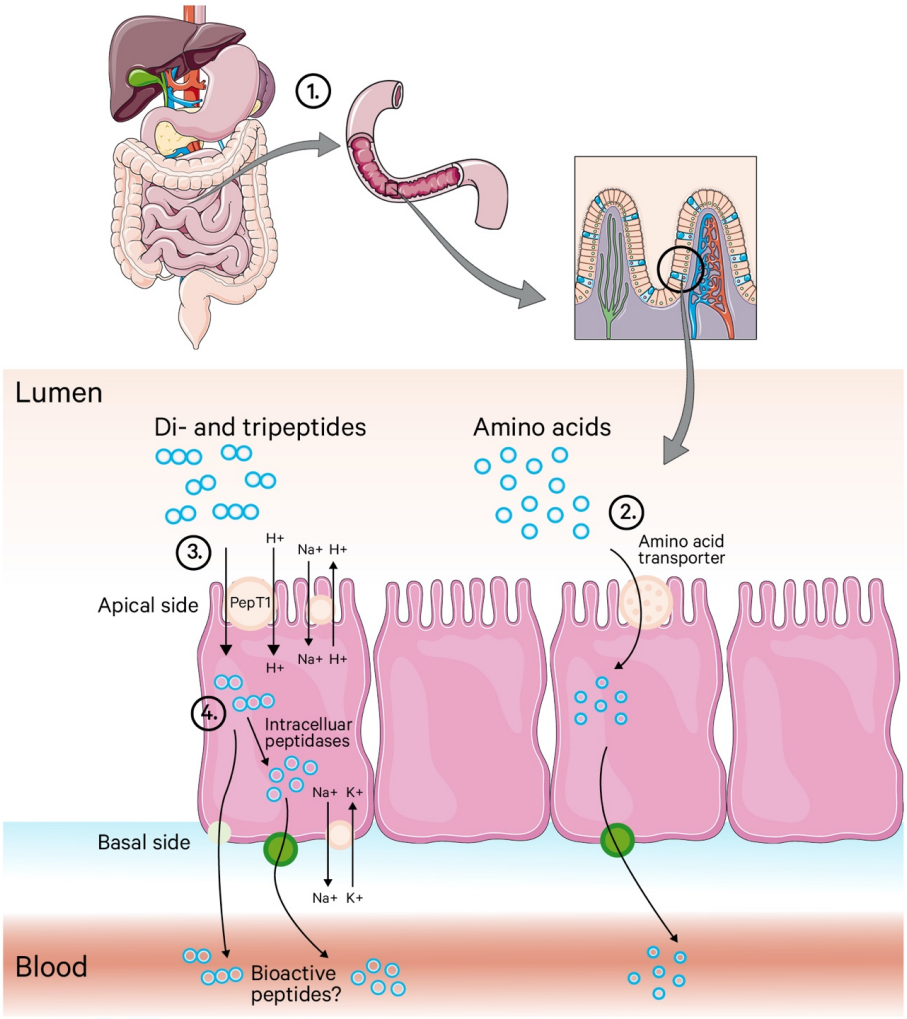

Figure 2. Digestion and absorption of proteins in humans. (1) Proteins are unfolded and cleaved into polypeptides in the stomach, followed by further digestion to short peptides and free amino acids in the small intestine. (2) Free amino acids are absorbed rapidly through amino acid transporters. (3) Di- and tripeptides are actively absorbed into the enterocyte through the peptide transporter PepT1, followed by (4) degradation to free amino acids and transfer to circulation. It is suggested that bioactive peptides is absorbed through the same routes as above, and released into circulation as intact di- and tripeptides. The medical images are taken from Smart Servier Medical Art. The figure is made in Adobe Illustrator by Jensen 2020.

1.2.6 Bioactive properties of fish protein hydrolysates

A bioactive peptide can be defined as a "*food derived peptide that in addition to their nutritional value exert a physiological effect in the body*" (193). These bioactive peptides can enter the circulatory system and have a specific physiological effect in cells or organs, or a local effect in the GI tract (194). They must be released from their inactive state in the parent protein, through either hydrolysis by digestive enzymes, microbial fermentation or from enzymatic hydrolysis in food processing (194, 195). They usually contain between 2 to 20 amino acids, and the sequence of the amino acids is crucial for the specific activity of the peptide (194).

There has been a growing interest in identifying bioactive peptides that may prevent or reduce the burden of chronic diseases, such as CVD and T2DM, with an increased interest in nutraceuticals and functional foods (195). Bioactive peptides originating from milk have been researched to a large extent, and there are available products on the commercial market today, e.g., with suggested antihypertensive effects (194). There is emerging evidence that proteins from fish contain bioactive peptides, being able to modulate physiological processes in the human body and contribute with a number of effects beyond their nutritional value as a source of energy and amino acids (196, 197). Bioactive peptides with inhibitory effects on ACE, a key enzyme involved in blood pressure regulation, was first reported in sardine meat and has later been identified in other fish species and fish protein hydrolysates made from residual raw materials (185, 194). Fish protein hydrolysates are also suggested to contain bioactive peptide sequences with antihypertensive, antioxidative and antimicrobial activity and glucose-regulating effects (185, 196).

1.3 RATIONALE

Given the increasing rates of overweight and obesity, and the concomitant increase in lifestyle-related diseases, such as T2DM, CVD, there is a need for effective strategies for treatment and prevention. Animal and clinical intervention studies have indicated that the intake of cod fillet may have beneficial effects on metabolic health, such as glucose and lipid metabolism. Results reported from animal studies investigating the effect of fish protein hydrolysate on metabolic outcomes show promising results (48-52). There are few studies in humans with discrepant findings (53-57), illustrating the need for more research, in particular if the potential beneficial effects observed in animal studies may be transferred to a human population. To our knowledge, no study has explored potential health effects of a low dose with a protein hydrolysate made from cod in a population with MetS.

As Norway is one of the major fishing nations in the world (73), we have a great responsibility to utilize the marine resources in terms of sustainability, environmental footprint, and value creation. Large amounts of residual raw materials are generated within the fishing and aquaculture industry every year, with 320 000 tonnes occurred within the whitefish sector in Norway in 2018 (183). These marine residual raw materials are rich in proteins, lipids and other valuable components, such as potential bioactive peptides. Despite increased utilization of these residual raw materials, around 40% is still discarded within this sector every year (74, 183). It is of interest to explore if these already harvested marine resources converted to products for human consumption, have the potential to improve human health by affecting metabolic markers of glucose and lipid metabolism, thereby creating new market opportunities for the industry and contribute to more sustainable industry.

2. OBJECTIVES

Main objective

The overall objective of the PhD project was to investigate if supplementation with a protein hydrolysate made by hydrolysis of residual material from cod fillet production, would have beneficial effects on glucose metabolism in healthy adults and on components of the MetS in overweight and obese adults.

The effect of supplementation with CPH was investigated in three clinical intervention studies. Two of the studies evaluated the effect of supplementation in healthy subjects, and the last study aimed to explore the effect in overweight and obese subjects with MetS. We hypothesized that dietary supplementation with CPH would beneficially affect glucose metabolism in the acute setting (Paper I), and that it was a dose-response relationship of increasing doses, with a plateau effect observed when increasing the doses (Paper II). Lastly, we hypothesized that supplementation with CPH for 8 weeks would beneficially affect components of MetS (Paper III), including adipokines and inflammatory parameters related to the syndrome (Paper IV).

Specific objectives:

Study 1: The aim was to investigate the response of supplementation with a low dose of CPH on glucose metabolism in healthy adults aged 40-65 years (Paper I).

Study 2: The aim was to investigate the response of supplementation with increasing doses of CPH on glucose metabolism in healthy adults aged 60-80 years, and to find a potential effective daily dose for further use (Paper II).

Study 3: The aim was to investigate the response of 8 weeks supplementation with a low dose of CPH on fasting and postprandial glucose metabolism, lipid profile, body composition (Paper III), as well as fasting levels of adiponectin, leptin, ghrelin and inflammatory parameters (Paper IV) in subjects with MetS.

3. MATERIALS AND METHODS

Papers I and II are based on Study 1 and 2, respectively, whereas Papers III and IV are based on Study 3. All three studies were conducted as multicentre trials at Haukeland University Hospital and Ålesund Hospital.

3.1 STUDY POPULATION AND DESIGN

3.1.1 Paper I

The study was a double-blinded, crossover study investigating the effect of a single dose of CPH on glucose metabolism. The study included two study visits, with a washout period of 4 to 7 days between the two visits. Participants received a drink containing 20 mg/kg body weight of CPH or casein (control) in randomised order. The primary outcome was postprandial response in glucose metabolism, measured by serum glucose, insulin and GLP-1 (Paper I). An overview of the study design is given in **Figure 3.**

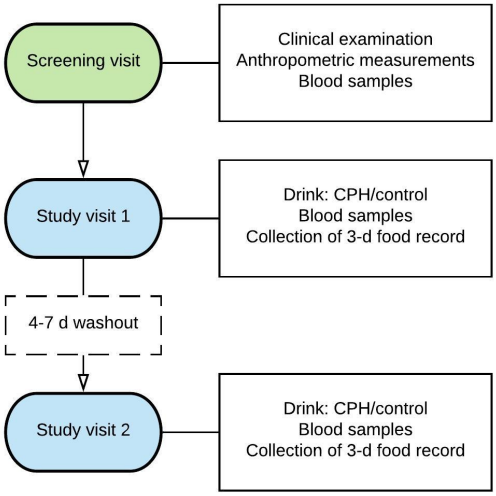

Figure 3. Overview of study design of Study 1 (Paper I). CPH: Cod protein hydrolysate, d: days.

Participants were recruited between October 2017 and February 2018, through an online recruitment form on the internet and posters at the two participating hospitals. Inclusion criteria were BMI between 20-30 kg/m^2, age between 40-65 years and signed informed consent. Exclusion criteria were allergy or intolerance to fish and shellfish, confirmed diagnosis of milk allergy, pharmacologically treated diabetes mellitus, low or fluctuating blood pressure, chronic diseases or therapies that could affect the evaluation of study end points, pregnant or lactating women, acute infections or unwillingness to comply with study requirements. Participants were not allowed to take any supplements containing long-chain n-3 PUFAs for one week prior to the first study day, and throughout the study. No change in eating habits or level of physical activity were allowed.

Participants indicating interest, were prescreened for general eligibility and compliance with inclusion and exclusion criteria by telephone. Eligible participants were invited for a further screening visit, including review of medical history and clinical examination by a physician, anthropometric measurements (height and body weight), measure of vital sign (BP, heart rate (HR)) and blood sampling. Included participants came to the research facilities on two identical study visits. The participants were given a standardised evening meal (rice, oatmeal or barley porridge), with instruction to eat this before 08:30 p.m. on the preceding day before each study visit, followed by fasting until the next morning. On the morning of the study visit, the participants came to their designated research unit in a fasting state between 08:00 and 09:00 a.m. A fasting blood sample were taken, followed by a drink containing CPH or control. Both drinks were a lemon-flavoured powder to be mixed with water and was served in neutral bottles. A standardised breakfast meal (test meal) was served immediately after the participants had finished the drink, with instructions to consume the meal within 15 minutes. Blood was collected in the fasting state, at 0 minutes (i.e., immediately after the test meal was finished), and thereafter at 20, 40, 60, 80, 100, 120 and 180 minutes. Blood pressure was monitored during the study visit (fasting state, 40, 80 and 180 minutes).

The standardised breakfast meal consisted of two slices of semi-coarse bread (50% whole wheat, 80 g weight), 10 g margarine, 20 g strawberry jam and 20 g white cheese. The drink contained 145 kcal and 35.9 g of carbohydrate. The meal provided a total of 500 kcal and 77 g carbohydrate.

3.1.2 Paper II

The study was a double-blinded, crossover study investigating the effect of increasing doses of CPH on postprandial glucose metabolism. The study consisted of four different intervention cycles, where the participants received a daily dose of CPH for one week, with one week washout period (7 days) between each dose interval. An overview of the study design is given in **Figure 4.** The dose intervals were 10, 20, 30 or 40 mg/kg body weight and assigned to the participants in a random order. The primary outcome was postprandial response in glucose metabolism, measured by venous samples of serum glucose and insulin. Secondary outcome was postprandial response in plasma GLP-1 (Paper II).

Participants were recruited between March and July 2018, and the study was completed between April and November 2018. An online recruitment questionnaire and advertisements on notice boards at the participating hospitals and external websites were used to recruit participants. Criteria for inclusion were age between 60-80 years, BMI between 20-30 kg/m^2 and signed informed consent. Exclusion criteria were the same as in Study 1, with the exception of milk allergy, and we excluded participants with excessive alcohol or drug consumption (assessed by a physician). We instructed participants to stop taking any supplements containing long-chain n-3 PUFAs for two weeks prior to the first study day, and throughout the study. No change in eating habits or level of physical activity were allowed.

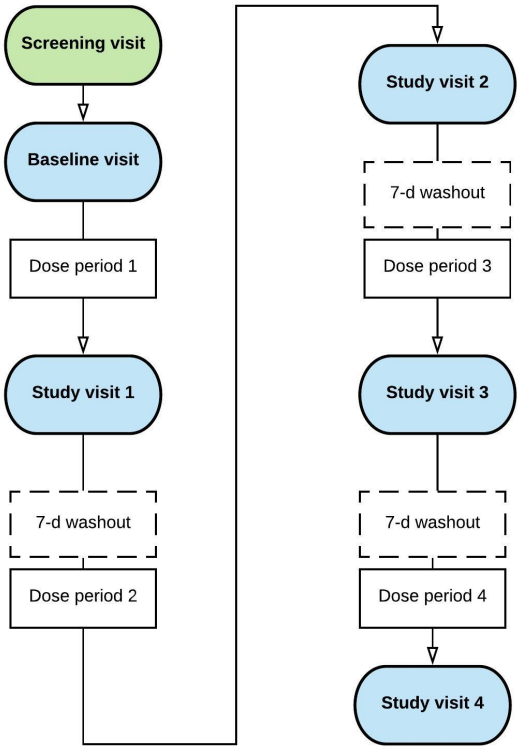

Figure 4. Overview of the study design of Study 2 (Paper II). Each dose period lasted for one week, and the participants came to the research facility on the last day in each dose period for a study visit. The total study period was 7 weeks. d: day.

Included participants came to the research facilities on six different occasions: one screening visit and five study visits. The screening visit included clinical examination and review of medical history by a physician, vital signs (BP, HR), anthropometric measurements (body weight and height) and blood sampling. At the first study visit (baseline study visit), the participants had not received any doses of CPH. This was included to assess baseline postprandial measurements before study commencement. Due to a methodological error, the results from this visit is not included in the manuscript of Paper II. Further description is given in subchapter 5.1.2.

The study consisted of four intervention cycles. The participants received six bottles containing powder with CPH, labelled 1 to 6, with written instruction to mix with water and consume before breakfast each morning in the intervention cycle. On the last day (day 7) of each intervention cycle, the participants came to the research facilities between 08:00 and 09:00 a.m. They came in a fasted state and had been instructed to not eat or drink anything (except water), and refrain from substances with nicotine from 09:00 p.m. the night before. At the study visit, a fasting blood sample was drawn from an antecubital vein. The participants were given the last dose of CPH in the current intervention cycle, followed by a standardised breakfast meal (test meal) 10 minutes after the drink. The first postprandial blood sample was taken 25 minutes after the drink was served and 15 minutes after the test meal (0-minute sample), followed by blood sampling every 20 minutes for 120 minutes (**Figure 5**).

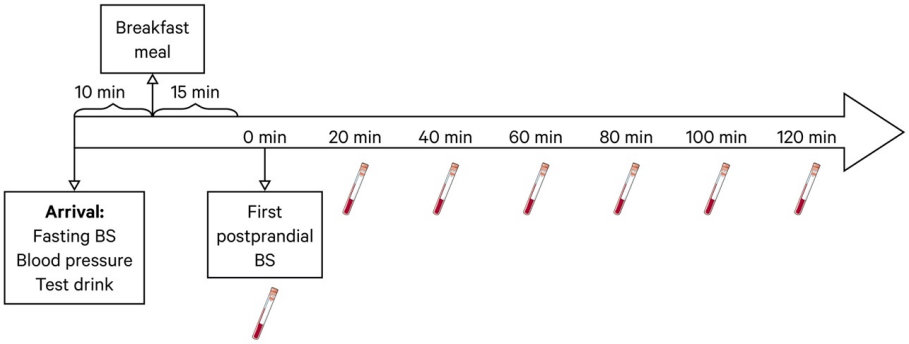

Figure 5. Timeline of the study visits in Study 2. All study visits were identical. Blood samples were taken every 20 minutes for 120 minutes. The first postprandial blood sample was taken 25 minutes after the drink was served and 15 minutes after the test meal.
BS; blood sample.

At the end of the study visit, six bottles for the next intervention cycle were handed out with instructions for when to start the new intervention cycles. A washout period of seven days was inserted between each intervention cycle. As a reminder, the participants received a text message before starting on the intervention cycle and before each study visit. The baseline visit was identical to the other study visit, but the participants were given a glass of orange juice instead of a CPH-containing drink.

The standardised breakfast meal was the same as used in the previous study, but the drink contained 22.5 g carbohydrate and 100 kcal. Overall, the test meal provided a total of 455 kcal and 63.5 g carbohydrate.

3.1.3 Papers III and IV

The study was a double-blinded, randomised intervention with a parallel-group design. Participants with MetS received a daily supplement of 4 g of CPH or placebo for 8 weeks. The primary outcomes were fasting and postprandial glucose levels (Paper III). Secondary outcomes were other metabolic and clinical parameters of MetS (WC, fasting TAG and HDL-C), fasting and postprandial levels of insulin, GLP-1 (Paper III) and acylated ghrelin, fasting levels of adiponectin, leptin and inflammatory markers (Paper IV).

Recruitment took place between March and September 2019, and the study was completed during three intervention periods: April-June 2019, August-October 2019 and October-December 2019. Participants were recruited through an online recruitment questionnaire, with advertisements on social media, notice boards at the participating hospitals and at general practitioners in Bergen and the surrounding area. Inclusion criteria were age between 40-70 years, BMI between 27-35 kg/m^2 and the presences of MetS. MetS was defined according to the JIS definition, where the presences of any three of five given risk factors qualifies for a diagnosis of MetS: elevated fasting serum glucose or serum TAG, reduced HDL-C, increased WC or elevated BP. Please see subchapter 1.1.1 and **Table 1** for the specific cut-off points used. For WC, we used the IDF cut-off points (WC:≥80 cm in women; ≥94 cm in men) (19). Exclusion criteria were the same as in Study 2. Since BP was not an outcome measure, we allowed participants using certain types of blood pressure medications, not known to clinically affect glucose metabolism, to be included. This included agents acting on the renin-angiotensin system, calcium channel blockers and diuretics, whereas participants using peripheral vasodilators or beta-blocking agents were excluded. The participants had to stop the use of nutritional supplements containing long-chain n-3 PUFAs for a minimum of four weeks prior to the study and during the intervention period. The participants could not be involved in any weight-

loss program and been weight-stable for 3 months prior to the intervention. No changes in physical activity level or food consumption were allowed. An overview of the study design is shown in **Figure 6.**

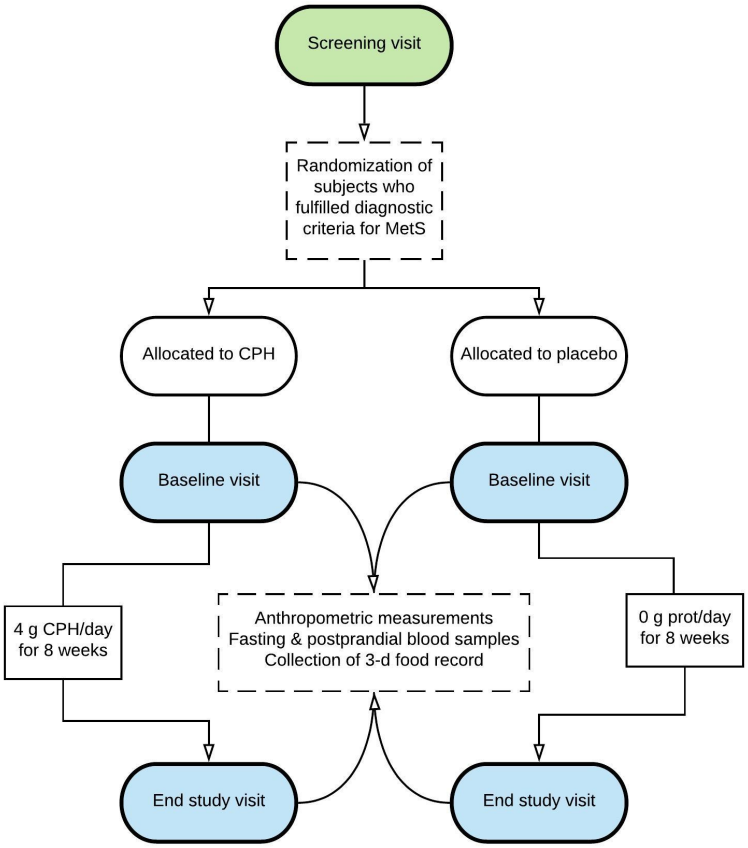

Figure 6. Overview of the study design of Study 3 (Papers III and IV). MetS: Metabolic syndrome CPH; cod protein hydrolysate.

We prescreened all possible participants that had responded through the online recruitment questionnaire by telephone. Potential participants were invited to a screening visit to evaluate the presence of MetS and eligibility to inclusion and exclusion criteria. The screening visit included a review of medical history and a clinical examination by a physician, measure of vital signs (BP, HR) and anthropometry (height, body weight, WC), and blood sampling. Included participants

met on two identical study visits: a baseline visit (prior to study commencement) and end of study visit (after 8 weeks of intervention). The participants came to the study centre in the morning, after an overnight fast (after 9:00 p.m. the previous day, the participants could not eat, drink or use nicotine). When they arrived, fasting blood samples were taken, followed by anthropometric measurements. Body composition was measured by a bioelectrical impedance analysis device at baseline and end of study visit. The subjects then consumed a standardised breakfast meal (test meal), providing a total of 440 kcal and 69 g carbohydrates. The meal had to be consumed within 15 minutes and was followed by blood sampling at 0 minutes (i.e., immediately after the meal was finished), and thereafter every 20 minutes until 120 minutes. Eight weeks supply (56 doses) of the prepacked test material (active or placebo) was handed out to the participants at end of the baseline visit. They started the intervention on the following day and were instructed to open the sealed bag with powder, mix well with 100 mL cold water and consume 10 minutes before breakfast, daily for 8 weeks.

3.2 PRODUCTION OF TEST MATERIAL

The test materials used in this PhD project was produced by Firmenich Bjørge Biomarin AS (Ellingsøy, Ålesund, Norway).

The fish protein hydrolysate was made by enzymatic hydrolysis of fresh frozen meat (cutting and trimmings) from Atlantic cod (*Gadus morhua)* obtained from the filleting industry. A batch of 500 kg frozen (-10°C) raw material was grounded, transferred to an incubator and mixed with sweet water at a ratio of 1:1. The mixture was stirred at 80 rpm and heated to 55°C, before adding the enzyme preparation Protamex® (Novozymes AS, Copenhagen, Denmark), followed by incubation for 45 minutes, at 55°C, 80 rpm and pH 7.0. The enzyme was added according to guidelines from the producer. To inactivate the enzyme, the preparation (incubate) was heated to 90°C for 15 minutes. The preparation was then passed through a rotating sieve (Swenco, Sweden) to remove any bone fragments. A two-phase centrifugation (Alfa Laval AS, Denmark) was used to separate the peptide-containing water-soluble fraction (the hydrolysate) from the indigested residue. This was followed by ultrafiltration and dehydration of the soluble phase to a 50% dry matter concentrate, which was spray-dried to a powder. The spray dried CPH powder contained 89% crude protein and <0.2% fat, 0% carbohydrate, <3.0% water, 10% ash, 0.1% NaCl, 1.7% sodium and 0.0% chloride, by weight. It was stored at 12°C in 10-kg diffusion tight packaging until used.

Table 3. The molecular weight profile of the cod protein hydrolysate used in the clinical trials included in the PhD project.

Molecular weight (Da)	Amino acid moieties	Percentage (%)
< 2000	< 18 or less	90 %
< 1000	< 10 or less	75 %
< 500	< 5 or less	55 %
< 200	≤ 2*	25-30 %

*Dipeptides and free amino acids. Da: Daltons

The molecular weight (MW) profile of the CPH was analysed by MW exclusion chromatography, with results showed in **Table 3.** Free amino acids accounted for

4.77% of the hydrolysate. The ratio between essential amino acids and non-essential amino acids was 0.70. The composition of amino acids and taurine of the CPH is given in **Table 4**. In Papers I and II, lysine has been highlighted as a branched chain amino acid (BCAA) in the tables (please see Table 2 in Paper I and Table 1 in Paper II). This is not correct, and valine should be highlighted as a BCAA.

In all three clinical trials, the CPH was provided to the participants in sealed packages containing a powder to be mixed with water. Further description of the different control or placebo materials used in the individual studies, are given in detail in each individual paper.

Table 4. Composition of amino acids and taurine of the cod protein hydrolysate used in the clinical trials included in this PhD project.

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 (BCAAs)

3.3 BIOCHEMICAL ANALYSES

In all three studies, baseline biochemistry for safety purposes, serum glucose and insulin and plasma GLP-1 were handled the same way.

We collected samples for safety purposes (albumin, prealbumin, glycated haemoglobin (HbA1c), leucocytes, thrombocytes, haemoglobin, sodium, potassium, alanine aminotransferase, alkaline phosphatase, creatinine and aspartate aminotransferase) at the screening visits and end of study visits. In Study 2 (Paper II), samples for determination of vitamin B12 and D were also taken to assess nutritional status. All tests were analysed according to standard accredited methods at Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital (Bergen, Norway), and the Department of Medical Biochemistry, Ålesund Hospital (Ålesund, Norway).

Samples for determination of serum insulin and glucose were collected in serum separator cloth activator tubes. Serum was obtained by centrifugation of full blood at 2000 g x at 20°C for 10 minutes, after 30-60 minutes of coagulation. Concentration of serum insulin and glucose were analysed according to standard accredited methods at Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital.

Samples for determination of GLP-1 were collected in Vacutte® EDTA-K2 tubes, with 20 µl DPP-4 added prior to sampling. Plasma was obtained by centrifugation of EDTA blood at 1800 x g at -4°C for 10 minutes, within 20 minutes of blood sampling. Plasma GLP-1 was analysed by the ELISA method, the specific details about the kit are given in the respective papers.

In Study 3 (Papers III and IV), samples of Hba1c, high-sensitive CRP (hs-CRP), total C, HDL-C, LDL-C and ratio of total C: HDL-C were analysed before intervention and at end of study. All these tests were analysed according to standard accredited methods at the same routine hospital laboratories as mentioned above. We also measured adiponectin, leptin, acylated ghrelin and inflammatory markers. Serum for analyses of

hs-CRP, other inflammatory markers, adiponectin and leptin were obtained by the same method as previously described for serum insulin and glucose. Serum adiponectin, leptin and ghrelin were analysed using ELISA kits, whereas the inflammatory cytokines were analysed by the Cytokine human ultrasensitive magnetic 10-plex panel. More details about the specific kits can be found in Paper IV. Samples for ghrelin determination were collected in Vacuette® EDTA Aprotinin tubes, added 34 µl 4-(2-aminoethyl) benzensulfonyl fluoride hydrochloride Ready-made solution to the tubes right after blood sampling. Plasma was obtained by the method as described for GLP-1 previously.

3.4 ESTIMATION OF DIETARY INTAKE

Participants in all three studies recorded their dietary intake using dietary records. Based on the reported intake from the participants, energy and macronutrient intake were estimated using "Kostholdsplanleggeren" (Norwegian Food Safety Authority and the Norwegian Directorate of Health, Oslo, Norway) (198). In Study 1 (Paper I), the participants filled out a 3-day dietary record prior to the first study visit, and a 1-day record prior to the last study visit. In Study 2 (Paper II) and 3 (Papers III and IV), the participants filled out a 3-day dietary record before study commencement and at end of study. In Study 2, the first seven participants also filled out a 1-day dietary record before each study visit, but this was omitted for the remaining participants due to the high burden on the participants.

The dietary records were used to evaluate the composition of the diet prior to intervention and assess whether any changes were made in the diet during the intervention period. In Study 1, we used the 1-day dietary record to compare estimated energy and macronutrient intake before each study visit. In Study 3 (Paper III), the caloric and protein content of the test material were added to the dietary records at end of study.

3.5 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS Statistics version 24.0 (Papers I and II) and 26.0 (Papers III and IV) (IBM Corp., Armonk, NY, USA). In Paper II, Stata v15.1 (StataCorp LLC, Texas, USA) was used to estimate the effect of dose using a mixed effects model. All graphical work was conducted in GraphPad Prism version 7.0 (Papers I and II) and 8.4.1 (Papers III and IV) (GraphPad Sotfware, Inc., San Diego, CA, USA). Data was evaluated for normality using the Shapiro-Wilk test and histograms, and non-normally distributed data was log-transformed before using parametric tests. Level of significance was set to $p < 0.05$.

3.5.1 Paper I

A linear mixed-effects model with repeated measures was conducted to evaluate the difference between CPH and control, the model was adjusted for gender. Insulin and GLP-1 were log-transformed before statistical testing. An independent samples *t*-test was used to compare total area under the curve (AUC) for postprandial glucose. A paired samples *t*-test was used to assess the difference between energy and macronutrient intake between the two different study days. It is stated in Paper I that a two-way analysis of variance with repeated measures was used to evaluate differences between each time point, this is an error in the publication and was not done.

3.5.2 Paper II

A paired samples *t*-test was used to detect changes in nutrient intake from baseline to end of study. To estimate the effect of dose, the maximum observed value and the AUC for the time course of each outcome variable were calculated for each combination of person and dose. We then fitted mixed models with the outcome measures (maximum value or AUC) as the dependent variable, fixed effects of dose and random intercepts across person. Carry-over effects were assessed using a standard likelihood-ratio test, to test for interaction between dose and ordering.

3.5.3 Papers III and IV

Paper III

The paired samples *t*-test was used to assess change from baseline to end of study within groups. An independent samples *t*-test was used to compare changes from baseline to end of study between the two groups, and compare AUC between groups for postprandial glucose, insulin and GLP-1. A linear mixed-effects model with repeated measures was used to examine group differences over time for the postprandial measurement of insulin, glucose and GLP-1. The GLP-1 data was log-transformed before performing the analysis.

Paper IV

A linear mixed-effects model with repeated measures was used to examine group differences over time for the postprandial measurements of ghrelin. For data not improved by log-transformation, non-parametric tests were used. The Wilcoxon's Signed Rank test was used to evaluate changes within groups, and the Independent Samples Mann Whitney U test was used to compare changes from baseline to end of study between CPH and placebo. A Pearson's correlation coefficient analysis was used to evaluate correlations between ghrelin and body weight, as well as ghrelin and BMI.

3.6 ETHICS

All three studies were conducted according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants in all studies prior to study commencement.

Study 1 (Paper 1): The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2017/1794). The trial was registered at www.clinicaltrials.gov as NCT03669796

Study 2 (Paper II): The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2017/1795). The trial was registered at www.clinicaltrials.gov as NCT03526744

Study 3 (Papers III and IV): The study was approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2018/2163). The trial was registered at www.clinicaltrials.gov as NCT03807752

4. RESULTS

4.1 PAPER I

Forty-one participants (26 women, 15 men) were randomized and completed the study. Eighteen participants were allocated to receive CPH drink and 23 were allocated to receive control drink at the first study visit. The participants received the opposite drink at the second study visit. The included participants had a mean age of 51±6 years with mean BMI 25.2±3 kg/m^2.

No statistically significant difference was observed in the estimated mean energy intake before each study visit. Mean fasting glucose and GLP-1 levels were numerical equal at both study visits, whereas the insulin levels were numerically higher before the CPH drink than control drink. The serum glucose concentration peaked 20 minutes after the first postprandial blood samples after both drinks, whereas serum insulin concentration peaked at 20 minutes after the CPH drink and 40 minutes after the control drink. The concentration of plasma GLP-1 peaked at the first postprandial blood samples (0 minutes) after both drinks.

The AUC calculated from the fasting through the postprandial test point did not reveal any significant differences in glucose when comparing CPH and control. A linear mixed-effects model with repeated measures did not reveal any differences between CPH and control in concentrations of glucose or GLP-1, but the insulin concentration was significantly lower after supplementation with CPH drink compared to control drink.

4.2 PAPER II

Thirty-three participants were enrolled in the study, and thirty-one (18 women, 13 men) completed the study. The participants were randomised to which order they would receive the different dose levels (10, 20, 30 and 40 mg/kg BW). Mean age was 67.8±4.9 and mean BMI was 26.0±2.6 kg/m^2.

No statistically significant difference was observed in estimated energy or macronutrient intake over the course of the study. When comparing the lowest dose of 10 mg/kg BW of CPH (reference category) with the higher doses (20, 30 and 40 mg/kg BW), no statistically significant differences in estimated maximum value of glucose, insulin or GLP-1 were observed. When the participants were given the highest dose compared to the lowest dose, the estimated maximum value of glucose, insulin and GLP-1 was 0.28 mmol/L, 5.14 mIU/L and 0.34 pmol/L lower, respectively. No statistically significant differences in AUC were observed for any of the outcome measures, when comparing the lowest dose with the higher doses (20, 30, 40 mg/kg BW). For glucose, AUC was calculated from t=baseline until t=80, excluding t=100 and t=120 minutes, based on the assumption that the glucose levels for the majority of individuals had returned to their baseline levels within 80 minutes. If all measuring points were included, the significance of the results did not change.

4.3 PAPERS III and IV

Thirty-one participants were randomised to receive CPH or placebo for 8 weeks. Fifteen participants were allocated to the CPH group and 16 allocated to the placebo group. Thirty participants completed the study and was included in the statistical analysis, with fifteen in each group. In the CPH group, mean age was 52.8±6.3 years and mean BMI 32.7±2.2 kg/m^2, with mean age of 53.4±6.8 years and mean BMI 32.4±3.3 kg/m^2 in the placebo group.

The estimated energy and macronutrient intake were similar between the groups at baseline, with no statistically significant changes observed during the study. We did not observe statistically significant changes in any of the anthropometric measurements during the study. Eight weeks supplementation with CPH did not affect fasting or postprandial serum concentrations of glucose, insulin, GLP1 or ghrelin, when compared to placebo. HbA1c did not change within the groups during the study, and there was no statistically significant difference between the groups after 8 weeks.

Fasting serum TAG concentration was significantly decreased within the CPH group after 8 weeks. When comparing the change in TAG levels from baseline to end of study in the CPH group to the placebo group, no statistically significant difference was observed. Fasting total C and LDL-C were statistically significantly decreased within both groups at end of study, with no differences between the groups. No statistically significant changes within or between groups were observed for serum concentration of HDL-C or in the ratio of total-C: HDL-C during the study.

Supplementation with CPH increased fasting serum concentration of adiponectin after 8 weeks. When comparing the change in fasting levels of adiponectin, no statistically significant difference between groups was observed. After 8 weeks, the median change in hs-CRP levels from baseline to end of study in CPH group was significantly different, compared to the placebo group. The concentrations of the other inflammatory parameters or fasting serum levels of leptin were not significantly affected by CPH supplementation.

5. DISCUSSION

5.1 DISCUSSION OF METHODS

5.1.1 Study populations

The protein hydrolysate used in the three studies summarised in this thesis, had not formally been investigated in an intervention study in humans previously. We therefore wanted to explore a potential effect of supplementation in healthy adults, with a presumed normal glucose regulation. Based on this, we included healthy normal- to overweight adults, with a BMI between 20-30 kg/m^2 in the first two studies (Study 1 and 2). In Study 1 (Paper I), we included participants between the age of 40 and 65 years, based on the assumption that many adults within this age group develop overweight and obesity, including metabolic disturbances (199). Favourable changes in glucose metabolism and other potential findings after the intake of a dietary supplement with CPH, are of relevance as it might affect development of metabolic disturbances such as hyperglycaemia and hyperlipidaemia. In the major perspectives, this may prevent or attenuate development of NCDs, such as CVD and T2DM.

The target group of Study 2 (Paper II) was healthy adults between the age of 60 and 80 years. The study was designed to establish an optimal dose level for use in further studies and future study protocols. We were also interested to investigate the effects of a dietary supplement with CPH on glucose metabolism in this age group, as previous studies have suggested impairments in glucose metabolism among older adults (200). A reduced capacity for glucose disposal is suggested to results from a reduced skeletal muscle mass observed in older adults (201). It was initially planned to further conduct a study with CPH-supplementation in older adults with loss of muscle mass and function, using the current study (Study 2) to determine the appropriate dose level.

In Study 3 (Papers III and IV), we investigated the effect of supplementation with CPH on components of MetS. We included overweight and obese adults with MetS, aged 40-65 years, with a BMI between 27 and 35 kg/m^2. The lower limit was set because we wanted to exclude potential participants that had a high BMI due to

increased muscle mass, and not due to increased fat mass. The upper BMI limit was set to exclude potential participants with an obesity grade II and above (≥ 35 kg/m^2), based on the assumption that these individuals were more likely to have comorbidities related to their obesity and use medications. It is possible that we lost potential participants with MetS due to this upper BMI limit.

5.1.2 Intervention and control materials

The cod protein hydrolysate

The CPH supplement used in the current project contained a large proportion of di- and tripeptides, hypothesized to be effective due to the presence of rapidly absorbable bioactive peptides. We hypothesized that these peptides affected glucose and lipid metabolism in a favourable way, through signaling pathways such as PI3K/Akt and mammalian target of rapamycin. The effect of CPH was explored using supplementation with low doses in all three studies. The scientific rationale for the use of these low doses are limited, and mainly based on unpublished and explorative data in animal and humans. An acute-effect study found improvements in postprandial insulin and glucose concentrations after supplementation with 20 mg/kg BW of CPH before a meal in 12 healthy male individuals, compared to whey and casein protein. Supplementation of CPH with the same dose level in nine healthy subjects, had beneficial effect on self-reported appetite and appetite hormones, with increased secretion of GLP-1 and peptide-tyrosine-tyrosine, and reduced levels of ghrelin (unpublished data from studies conducted at Clinical Trial Consultants AB, University Hospital, Uppsala University, Sweden provided by Einar Lied, former scientific advisor for Firmenich Bjørge Biomarin AS). It was of interest to explore these indications of possible beneficial metabolic effects on glucose metabolism. There is also some previously published studies reporting favourable metabolic effects after supplementation with low doses of fish protein hydrolysates in humans (56, 57), and a previous study in overweight and obese adults reported beneficial effects after supplementation with 3-6 g/day of unhydrolysed cod protein for 8 weeks (76). Based on this background, we chose to use a weight-adjusted low dose of 20 mg/kg BW as the starting dose in Study 1 (Paper I).

In Study 2 (Paper II), we investigated the effect of different dose levels on glucose metabolism. We chose the lowest dose of 10 mg/kg BW, to explore if it was possible to observe any effect on glucose metabolism at a lower dose level compared to the dose used in Study 1 (20 mg/kg BW). We were also interested in investigating if a more pronounced metabolic effect could be observed by increasing the doses, and if we eventually reached a plateau effect at the highest dose of 40 mg/kg BW. Based on the results from Study 2 (Paper II) and previously reported studies using low doses of cod protein (76, 78), we chose to use the highest dose of 40 mg/kg BW as a starting point in Study 3 (Papers III and I). As these participants received daily doses for 8 weeks and we intended to include 60 participants, it was considered too demanding logistically to weight-adjust the doses to the individual participant. We therefore used a fixed dose of 4 g (corresponding to 40 mg/kg BW in an individual weighing 100 kg). Overall, the dose levels of CPH investigated in the current PhD project might have been too low to be able to observe any significant metabolic effects, which may in part explain the lack of findings.

In all three studies, the CPH supplement was provided to the participants as a powder to be mixed with water. A potential challenge with a hydrolysate made from cod is the potential taste and smell of fish, and bitter taste originating from the hydrolysate, making it challenging to achieve double blinding. The taste and smell were masked by adding lemon flavour, by Firmenich Bjørge Biomarin AS, a company partly specialized within artificial fragrance and flavour. We could have provided the intervention material to the participants as tablets or capsules. This had been tried by Firmenich Bjørge Biomarin AS previously, resulting in a very distinct fish odour. Considering that this would make it difficult to achieve blinding, we chose to use a powder to be mixed with water.

Control (placebo) materials

Casein was chosen as control material in Study 1 (Paper I). The control material was provided to the study participants as a powder to be mixed with water, in the same dosage as the CPH supplement (20 mg/kg BW). We wanted an isonitrogenic and isocaloric control material. The protein content of the control material was in form of an intact protein (not hydrolysed protein), which is the main difference between the active material (CPH) and the control material. The use of casein as control material may be considered a limitation to the study design, as studies have reported beneficial effects on glucose and insulin response when high doses of casein have been added to a carbohydrate load (202). However, low doses of hydrolysed casein (<6 g added to a carbohydrate load of 50 g) do not seem to affect glucose metabolism (203). Considering that casein was provided in very low doses, on average 1.5 g in the study group, and as an intact protein, we assume that casein did not affect the results in Study 1. However, we cannot rule out the possibility that the proteins present in the control material have influenced the glucose metabolism and possibly masked an effect of CPH. A control material only containing glucose, with no protein, could have been a better choice. A limitation with this approach is that it would have been difficult to know if a potential effect was observed due to differences in nitrogen and energy content, or a "real" difference due to the protein hydrolysate. It might have been more appropriate to describe casein as a "positive control", and the study would have been strengthened by adding a control group that received no protein.

In Study 2 (Paper II) the participants received a glass of orange juice (2 dl) as a control drink. This was chosen for two reasons: 1) we wanted to compare the effect of the different doses against the effect of no protein, 2) facilitate an iso-caloric breakfast meal. A better choice might have been a control material consisting of a powder identical to the doses with CPH, but with no protein. The lack of protein would be the only difference to the active test material (CPH), making it more comparable to the other dose levels. This should be considered in future studies. The participants received the control drink at the baseline study visit, intended to be used as a control visit, with baseline postprandial measurement of insulin, glucose and GLP-1. At this

study visit, the participants were served the control drink with the breakfast meal at the baseline visit, while the drinks with test materials were served 10 minutes before the breakfast meal at the four study visits. When comparing the postprandial curves for glucose, insulin and GLP-1, the curve for the control visit is shifted to the right, with a delayed peak, compared to the four study visits during the intervention period. This is illustrated in **Figure 7**. We believe the shift is simply due to the fact that the drink at the control visit was given at a later time point compared to the test drinks at the four study visits, resulting in a delayed postprandial response at the control visit. This mistake was discovered after the study was completed, and we could not use the results from the baseline visit as a "control" for the other visits. This "control visit" is therefore not reported in Paper II, and the lowest dose level was chosen as a reference category. It was discussed to include a control day the first day of each intervention cycle, which would have strengthened the design, but it was considered too time consuming for potential participants.

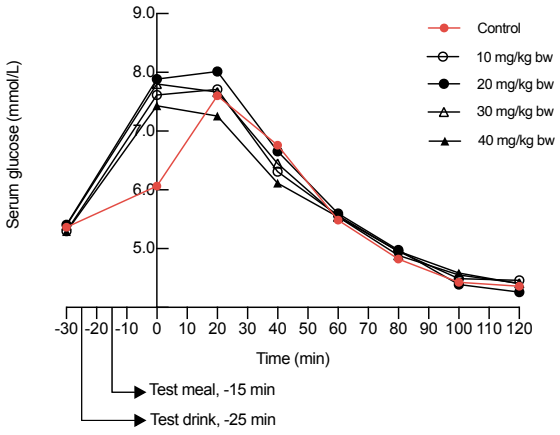

Figure 7. Postprandial curves for glucose concentrations after control visit (baseline visit) and four intervention cycles. At the control visit the participants received a glass of orange juice (control) together with the standardised breakfast meal, while at study visits they received the test drink 10 minutes before the standardised breakfast meal at study visits during the intervention (*n*=31). Bw; body weight.

In Study 3 (Papers III and IV), maltodextrin, an easily digestible polysaccharide extracted from corn starch, was used as placebo material. This was chosen because we wanted to evaluate the effect of CPH against the effect of no protein-supplementation. The placebo material contained 6.5 g maltodextrin, 5 g glucose hydrate and flavourings, whereas the intervention material contained 4 g CPH, 2 g maltodextrin and 5 g glucose hydrate, in addition to flavouring. The amount and name of the various flavourings used in the intervention and placebo material are given in detail in Papers III and IV. The active material (CPH) had a lower content of maltodextrin to achieve an iso-caloric supplement to placebo, as the protein content of the active material added some calories to the supplement. Overall, the placebo material contained 46.5 kcal, with 43.9 kcal in the active material. This small difference in caloric content is negligible and is not expected to have caused any impact on the results of Study 3.

5.1.3 Test meals

In all three studies, the participants consumed a standardised breakfast meal (described in subchapter 3.1.1-3) followed by blood sampling, to evaluate changes in postprandial glucose response. This breakfast meal, or test meal, resembled a typical Norwegian breakfast meal, containing a mix of carbohydrates, proteins and fats. Similar test meals have been used in other studies evaluating the effect of fish intake on glucose and lipid metabolism (71, 72, 78).

A mixed meal was chosen to evaluate the effect of CPH supplementation on postprandial glucose regulation after the consumption of a meal similar to a regular meal. This test meal was used as an alternative to the oral glucose tolerance test (OGTT). An OGTT is used to evaluate the glucose and insulin response after the intake of a standard amount of glucose (75 g) dissolved in water (204). The test is easy to reproduce and requires fewer resources (204). Compared to the OGTT that provides one nutrient, the test meal given to our participants might be more physiologically relevant as it resembles a normal meal and consists of a mixture of macronutrients, including proteins and fats, known to also affect glucose regulation (205). Since there is no general standard on how to compose such a mixed test meal, the energy content and composition of macronutrients will depend on the choice of food items included. This makes the comparison to other studies and physiological settings more difficult, which is a disadvantage of the test meals used in our studies. Another limitation is that the carbohydrate content of the test meals were different in the three studies, containing 77 g carbohydrates in Study 1, 63.5 g in Study 2 and 69 g in Study 3, making it difficult to compare the studies. The difference in carbohydrate content was due to different composition of the active (CPH) and control materials used in the three studies.

Weight-adjusting the test meal to each individual participant would have strengthened the study design, in line with the weight adjustment of intervention doses. This may be especially relevant in Study 3, where overweight and obese subjects were included, with a mean body weight at inclusion of 94.5 kg, but ranging from 74 kg to 133 kg. It is plausible that a participant with a body weight of 75 kg has a different glucose

response after a test meal, compared to a participant with a body weight of 130 kg. Weight-adjusting the test meals was discussed when planning the different studies. It was chosen not to be implemented in the study protocol, as it would have made the preparation of the test meal and implementation of study visit very time- and resources consuming.

We cannot rule out that proteins present in the test meal were competing with peptides present in the hydrolysate for uptake. This is particular an important point in Study 1 (Paper I), where the participants only received one single dose of CPH, but also relevant in Study 2 (Paper II). The test drink was given to the study participants 10 minutes before the test meal in Study 2, to avoid potential competition for uptake between the peptides present in the drink and proteins in the meal. This would have been preferable to also do in the first study, strengthening the design, but it was not done. Even though the test drinks were given earlier, there is no guarantee for uptake, and we do not know if the peptides present in the drink are preferred over other hydrolysed protein present in the small intestine. A limitation with providing the test drink 10 minutes before the test meal, is the long time period from the drink was ingested until the first postprandial blood sample was taken. In Study 2, the first postprandial blood sample was taken 25 minutes after ingestion of the drink (illustrated in **Figure 7**, subchapter 5.1.2), which may have caused us to miss the peak level of glucose, insulin or GLP-1.

5.1.4 Evaluation of dietary intake

Dietary intake was assessed in all three studies by using dietary records. This is a method of assessing dietary intake, where the intake of all foods and beverages consumed over a specified period are recorded as they are consumed (206). This method provides detailed information about the intake and is not prone to recall bias, but it is time consuming, may affect food selection and will not capture food items that are less frequently eaten (206).

In Study 1, the participants filled out a 3-day dietary record at baseline, prior to the first study visit, and a 1-day dietary record before the last study visit. A 3-day dietary record was not filled out at the end of study, due to the short time period between the two study visits (4 to 7 days). We expected that there would not be any major changes in the participants' dietary intake within this short period. The main purpose was to compare the energy intake prior to each study visit, to control that there were no major differences when comparing the two visits.

In Study 2, the participants filled out a 3-day dietary record at baseline and end of study. Given the long intervention period in total (7 weeks), it would have been an advantage to also have a dietary record during this period to assess that no major changes in nutrient intake were made during the intervention period. Ideally, the participants should have filled out one dietary record within each dose period. This would have enabled us to evaluate if any major changes in energy and macronutrient intake were made during the different dose levels, possibly influencing the results. To decrease the burden on the participants in the study, this was not included in the study.

In Study 3, the participants filled out a 3-day dietary record at baseline and end of study (8 weeks). The study design could have been strengthened by including a dietary record during the intervention period, e.g., after 4 weeks. However, these dietary food records might not be representative for the total intervention period, and relies on self-reported dietary intake, which has its limitations (207).

5.1.5 Measurement considerations

Several different anthropometric measurements were made, including height, body weight and WC, and body composition. Trained health personnel carried out all measurements to increase data reliability. Nevertheless, a limitation is that different study personnel performed the measurement at the two study sites. This might in particular have influenced the WC measurements in Study 3 (Paper III). In general, it can be challenging to measure WC, in particular in obese individuals (208). We measured WC according to WHO recommendations (209, 210), reported to be better correlated with abdominal obesity compared to e.g., waist-hip-ratio (208).

5.1.6 Power analysis

A power calculation was not performed for any of the three studies, due to lack of relevant data to base a power calculation upon and knowledge regarding what effect to expect when supplementing with low dose of CPH in humans. When we planned Study 1 (acute-effect study), the available evidence regarding low doses of cod protein in humans was studies using either the whole fillet (68, 72) or more long-term use of unhydrolysed cod protein (76). Based on the available evidence, we decided to include between 30 to 40 participants, as done in previous and comparable studies with cod protein (68, 76).

In Study 3, we planned to recruit 60 participants in total. We did not reach our target population size due to difficulties with recruitment of eligible participants. Due to limited resources and time constraint, we had to stop the inclusion of new participants in September 2019. Since no power calculations were done for any of the studies, it is possible that we did not include enough study participants to be able observe an effect.

5.2 DISCUSSION OF MAIN RESULTS

The majority of the global population live in countries where overweight and obesity pose a greater threat for society, as compared to underweight (92). Despite considerable effort from health authorities and the global community, the rates of overweight and obesity are increasing and there is no country to date that has been able to reverse this trend (99, 100). MetS has become a common metabolic disorder, as a result of the increasing rates of obesity. Obesity has great impact on public health, through increased costs and pressure on the health care system, reduced quality of life and premature deaths, and there is a need for effective prevention and treatment strategies (99, 100).

We conducted three intervention studies in the current project, summarised in this thesis, with the overall purpose of exploring if a dietary supplement with CPH could beneficially affect components of MetS, with a special focus on glucose and lipid metabolism. In the first study we explored the effect of a single low dose of CPH on glucose metabolism in healthy adults (Paper I), followed by a study investigating the most effective dose level of CPH for use in future studies (Paper II). The main results from these studies, reduced postprandial serum insulin concentration after a single dose of CPH (Paper I) and indications that a higher dose (40 mg/kg BW) of CPH is more effective on metabolic outcomes (Paper II), formed the basis of the last study (Study 3). In this study, we explored the potential metabolic effects of daily supplement with 4 g of CPH on components of the MetS in overweight and obese subjects. We observed that a low dose of CPH did not significantly affect parameters of glucose or lipid profile, inflammatory markers or body composition in individuals with MetS after 8 weeks (Papers III and IV). The main results will be discussed in the next subchapters.

5.2.1 Glucose metabolism and effects of cod protein hydrolysate

A daily supplement with 4 g of CPH for 8 weeks in individuals with MetS did not influence glucose metabolism, and we observed no statistically significant differences in fasting or postprandial levels of glucose or insulin when comparing the CPH group to the placebo group (Paper III). In agreement with these findings, we did not observe any effects on glucose concentrations after a single dose of CPH (Paper I) or with increasing doses of between 10-40 mg/kg BW (Paper II). However, we did observe a significantly reduced postprandial insulin concentration in Study 1 (Paper I). This is in contrast to previous studies with low doses of fish protein hydrolysate, reporting of no effect on insulin concentrations (53, 54, 57). It is debatable whether lower postprandial levels of insulin are beneficial in otherwise healthy adults. In our study, the results might indicate a more efficient utilization of insulin, since the glucose levels did not significantly differ between the two study visits and the participants exhibited a normal postprandial glucose response after CPH. The difference in insulin concentration between the CPH and control group are modest (please see Figure 3 in Paper I for a graphical representation of the insulin response), and the clinical significance of these findings is uncertain. Since we did not observe any effect on fasting or postprandial serum levels of insulin in the two following studies (Study 2 and 3), this further indicates that the observed decreased postprandial insulin levels could be a random finding. These results emphasize the importance of addressing the clinical relevance of the data.

The understanding and mechanisms of why low doses of CPH may beneficially affect glucose metabolism in the human body, are not fully understood. A greater rate of insulin-stimulated glucose uptake and increased insulin sensitivity have been observed in cultured L6 myocytes exposed to a cod protein-derived amino acid mixture, compared to cells exposed to casein- or soy-derived amino acid mixtures (66). It has been suggested that specific cod-derived amino acids, either individual or groups, directly affects the insulin-stimulated glucose uptake in the skeletal muscle cells, resulting in improved insulin sensitivity (66). This is suggested to be mediated through insulin activation of the PI3K/Akt pathway and translocation of GLUT4 to the skeletal

muscle cell (67). In obese rats fed a high-fat diet containing either casein or soy, the ability of insulin to activate PI3K was severely impaired, which was prevented in high-fat rats fed a diet with cod protein. The normalisation of the PI3K/Akt activation by insulin, was linked to an increased number of GLUT4 in the transverse tubules, indicating improved translocation of GLUT4, and thereby improved glucose uptake in the skeletal muscle cell (67). The content of BCAA in cod protein have also been implicated to be involved, by affecting signalling pathways such as PI3K/Akt and mammalian target of rapamycin, and thereby beneficially affecting glucose metabolism (211). The hypothesized effect of the CPH used in our studies, has been presumed to be due to specific cod-derived bioactive peptides present in the supplement. Bioactive peptide sequences have been identified in various fish species, suggested to be effective through inhibited DPP-4 activity and enhanced secretion of GLP-1 and cholecystokinin, beneficially affecting glucose metabolism (139, 196). We do not presume it is the protein intake *per se* causing an effect, since the CPH supplement only make up a small proportion of the total daily protein intake. For example, in Study 3, the estimated average daily protein intake for the participants receiving CPH was 85g/day, with the supplement accounting for only 4.7% of the total daily protein intake. We did not analyse for the presence of specific known bioactive peptide sequences in our hydrolysate, so the mechanism of action is therefore only a hypothesis.

Overall, the lack of findings on glucose metabolism might suggest that the different dose levels used in the three studies were too low to affect glucose metabolism, suggesting using higher peptide doses in future studies. In Study 1 (Paper I), there is a possibility that the control material (casein) may have masked a potential effect of CPH on glucose metabolism and influenced our results. In this trial, the study participants were healthy, normal- to overweight adults with no known abnormalities of the glucose metabolism. A single low dose of CPH prior to postprandial measurements, might therefore not result in any major effects on glucose levels. It would be reasonable to assume that a more pronounced effect could have been observed in overweight and obese subjects, as this group might exhibit abnormalities in glucose metabolism. Beneficial effects on glucose levels and improved glucose

tolerance have been observed in healthy overweight and obese individuals after 8 weeks supplementation with a low dose of cod protein (intact) (76). However, we did not observe any significant effects on glucose metabolism after CPH supplementation in individuals with MetS, in line with other studies reporting findings from studies with low doses of cod protein, ranging from 2.5 to 6 g per day, in healthy overweight and obese individuals (77, 78).

In contrast to our results from Study 3, previous studies exploring the effect of dietary interventions on metabolic risk factors in individuals with MetS have shown beneficial effects on glucose metabolism after various dietary interventions. A recent meta-analysis including 10 RCTs in patients with MetS, observed significant improvements in fasting glucose levels after multifaceted supervised lifestyle interventions (31). The lifestyle interventions consisted of various dietary interventions, targeting either healthy eating or caloric restriction, combined with supervised group-based exercise. The dietary interventions consisting of caloric restrictions targeting weight loss, including studies with longer intervention period and more supervised exercise, were more effective and resulted in greater improvements in the metabolic risk factors. It should be noted that the included studies had a low to moderate methodological quality (31). Furthermore, several dietary interventions with alterations in the macronutrient composition of fat and carbohydrates have reported beneficial effects on components of the metabolic syndrome, including glucose metabolism (212). A recent study observed that both very-high fat low carbohydrate diet and low-fat high carbohydrate diet resulted in improved levels of insulin, insulin C-peptide and Hba1c after 12 weeks intervention in individuals with an increased BMI and WC, irrespective of diet (213). Other proposed dietary strategies with effects on MetS, are energy-restricted diets, diets with a moderate to high protein intake and the Mediterranean diet (214). An intensive lifestyle intervention for 12 months in patients with MetS, combining an energy-restricted Mediterranean diet, increased physical activity and behavioural support, resulted in significant improvements in glucose metabolism, with reduced levels of fasting glucose, insulin and Hba1c (133). Some of the beneficial effects on glucose metabolism might be explained by the moderate weight loss observed in the intervention group (133). Compared to our study (Study 3), the

intervention period of this study is longer, the participants received an energy-restricted Mediterranean diet combined with increased physical activity and close-follow up, while we instructed our participants to not change their diet or level of physical activity. It is plausible to assume that the observed weight loss may have mediated some of the improvements seen on parameters of glucose metabolism. We did not observe any weight loss during the intervention. Our lack of beneficial effects on glucose metabolism in individuals with MetS, may also suggest that supplementation with a low dose of fish protein hydrolysate is not able to reverse changes that have already occurred in glucose metabolism of individuals with MetS. It would be interesting to investigate the combined effect of a low dose of CPH with a dietary intervention aiming at weight-reduction. It should also be noted that the sample size in Study 3 might have influenced our results, with too few subjects included in the study to be able to observe any effect on glucose metabolism. A full-scale study is needed to further evaluate potential effects.

5.2.2 Lipid metabolism and effects of cod protein hydrolysate

In Study 3 (Paper III), we observed a statistically significant reduction in fasting serum concentration of TAGs within the CPH group. These results are in agreement with observational studies reporting reduced levels of serum TAG after lean fish consumption (24, 46). Improved levels of TAG have also been reported after the intake of a diet containing lean seafood (88), and after a daily consumption of cod for two weeks (89). In contrast to these studies, a daily intake of 100-150 g of lean fish had no significant effects on TAG-levels in normal-weight (71) or overweight adults (90), or patients with MetS (47). It is suggested that the high content of taurine and glycine found in fish may contribute to increased excretion of faecal cholesterol and bile, thereby improving lipid variables in plasma (50, 75, 215). The improved TAG levels observed in some of these studies might also be caused by an increased fish intake replacing other major protein sources, such as meat, thereby affecting the intake of saturated vs. unsaturated fats. In all of these studies, the participants received fish in the form of whole fillet, which is a major difference compared to our study, providing only a small dose as a supplement.

In a more similar study to ours, by Hovland et al. (77), no significant effect on the TAG levels or any of the other measured lipid parameters were observed after 8 weeks with a daily supplement of 2.5 g of cod protein in overweight and obese individuals (77). In addition to using a lower dose compared to our study, they also used an intact protein. We have to interpret our results with great caution since the significant reduction of TAG only was observed within the CPH group. However, our findings might indicate that the protein hydrolysate contain some bioactive peptides released during the hydrolysis process contributing to the improved TAG levels. As also mentioned in subchapter 5.2.1, this is only a hypothesis since we did not test for the presence of known-lipid lowering bioactive sequences prior to starting the project. In comparison, no significant effects on postprandial TAG levels were observed in an acute-effect study after a single dose of 3 g fish protein hydrolysate made from bonito given to overweight and obese subjects (54). Since this study only gave a single dose to their participants, and used a fish protein hydrolysate made from another fish

species, it might suggest that length of intervention period and type of fish protein hydrolysate may be of significance.

In regard to dietary strategies observing improved effects on lipid markers in individuals with MetS, such as energy-restricted diets, diets rich in long-chain n-3 PUFAs and the Mediterranean diet, the improvements may be related to the concomitant weight loss observed in many of these interventions (214). An improved lipid profile, with elevated levels of TAG and reduced HDL-C were observed in patients with MetS, following an intensive lifestyle intervention, combining an energy-restricted Mediterranean diet, increased physical activity and behavioural support (133). They observed a moderate weight loss in the intervention group, which might explain some of the improvements observed in the lipid parameters (133). A recent meta-analysis of 9 RCTs found that lifestyle interventions, consisting of a combined dietary intervention and exercise, moderately reduced the levels of TAG by an average of 0.20 mmol/L, compared to usual care (31). Dietary supplements containing long-chain n-3 PUFAs taken for at least 3 months in doses above 1 g, have been reported to significantly reduce TAG levels with 7-25% (216). In line with these results, we observed a reduction of 18% in TAG levels from baseline to end of study within the CPH group (Study 3, Paper III). As previously mentioned, since the results were only reported within the CPH group, and not when compared to the control group, the results should be interpreted with caution. Overall, the potential effect of a low dose of a fish protein hydrolysate on lipid metabolism is of interest and should be evaluated in future studies with larger sample size.

5.2.3 Adipokines, inflammatory parameters and effects of cod protein hydrolysate

There are indications that lifestyle interventions may influence adiponectin levels, with energy-restricted diets combined with increased physical activity resulting in increased levels of adiponectin (131). It seems like the concomitant weight loss during intervention is of more importance for the increased levels, compared to type of dietary intervention (131). In line with these findings, male individuals with MetS consuming a Mediterranean diet combined with weight loss had increased adiponectin levels, with no significant effect observed in the absence of weight loss (132). These findings also seem to apply to leptin, with reduced levels observed as a result of weight loss (132, 133). The intake of fish and long-chain n-3 PUFAs supplements are also shown to increase adiponectin levels (131).

Interestingly, we observed that a daily intake of 4 g of CPH for 8 weeks in individuals with MetS significantly increased fasting serum concentration of adiponectin within the CPH group, without any significant changes in body weight or body composition (Study 3, Paper IV). We did not observe any significant changes in serum concentration of leptin. These results are similar to a previous study reporting increased levels of adiponectin after supplementation with 2.5 g of cod protein (intact protein, within group) in healthy overweight and obese subjects, with no change in leptin levels (77). Overall, these findings implies that cod protein in low doses may also be capable of increasing levels of adiponectin. When comparing the baseline fasting levels of adiponectin in our study, to previous studies in healthy adults (71) and overweight adults (77), the levels are lower and similar, respectively. This supports evidence suggesting that adiponectin levels are reduced in overweight subjects (121). Since we only observed increased levels of adiponectin within the CPH group, and not when comparing the groups, the results should be interpreted with caution. Still, the findings are of interest and should be further explored in future studies with larger sample size.

A recent meta-analysis comparing the effect of different dietary approaches on inflammatory markers in patients with MetS, observed that a low-fat diet resulted in reduced CRP levels (217). However, the effect appeared to be dependent on weight loss (217). Furthermore, it has been suggested that energy-restricted diets may improve the pro-inflammatory status observed in patients with MetS (214). An intensive lifestyle intervention in patients with MetS resulted in reduced levels of IL-18 and MCP-1, with only minor non-significant effect on hs-CRP, IL-8, IL-6 and TNF-α (133). It is suggested that the significant reductions in IL-18 and MCP-1 might be explained by the concomitant weight loss observed in the intervention group, including increased activity level and dietary changes (133).

Fish as part of the diet is also suggested to have anti-inflammatory properties, and reduced levels of CRP have been linked to consumption of fish (112). Insulin-resistant subjects receiving a diet with cod protein had reduced levels of hs-CRP after 4 weeks (113), with no effect on CRP levels after a high intake of cod in either normal-weight (71) or overweight (72) adults. In our study (Study 3), we found higher levels of hs-CRP after supplementation with CPH for 8 weeks, when compared to placebo (Paper IV). These results are in line with a study reporting of increased CRP levels after supplementation with 5.2 g of fish protein hydrolysate, however, in a population of older nursing home residents (53). In contrast, a low dose of cod protein did not affect CRP levels in overweight and obese subjects (76). Our findings are difficult to interpret. It is plausible that the small sample size might have influenced our results, or that some individuals may have had on-going low-grade inflammation without disclosing a problem. When comparing the overall fasting level of hs-CRP in both groups in our study with a previous study (218), our levels are similar to the levels reported in obese subjects and higher compared to normal-weight subjects, emphasizing how BMI may affect CRP levels (218). These results support findings suggesting that elevated levels of CRP may be involved in MetS (219, 220).

6. CONCLUSIONS

A daily supplement of 4 g CPH for 8 weeks, did not significantly affect parameters of glucose or lipid metabolism, inflammatory markers or body composition in overweight and obese subjects with MetS, when compared to placebo. Furthermore, we did not observe any effects on fasting levels of adipokines or fasting or postprandial ghrelin concentration. Considering the small sample size, no definitive conclusion can be drawn from this study. The tendency of beneficial effect on some lipid parameters and adipokines should be investigated in future studies.

An acute-effect study showed that supplementation with a dose of 20 mg/kg BW of CPH before a meal test, decreased postprandial insulin concentrations, with no effect on glucose or GLP-1 levels, in healthy adults between 40-64 years old. The reduced insulin levels are of uncertain clinical significance, and given the small population, these findings need further investigations.

No effect was observed on concentrations of serum glucose, insulin or GLP-1 levels with increasing doses of CPH, ranging from 10 mg/kg BW to 40 mg/kg BW, given to adults between 60 and 80 years. The results indicate a tendency that the highest dose of 40 mg/kg BW, corresponding to 3 g in an individual weighing 75 kg, was the most effective in lowering postprandial insulin and glucose levels. These are only indications, also with a need for further investigation.

Overall, our findings do not support our hypothesis that a low dose of CPH would beneficially affect glucose metabolism in the acute setting, and that it was a dose-response relationship of increasing doses with a plateau effect observed when increasing the doses, and that supplementation with CPH would beneficially affect components of MetS. Despite lack of findings and clear conclusions, due to preliminary findings, small populations and a number of limitations addressed in the thesis, these results add valuable information to the scientific field.

7. FUTURE PERSPECTIVES

The clinical studies, summarised in the current PhD thesis, were designed to investigate the potential effects of a low dose of protein hydrolysate made from residual material from cod fillet production on glucose metabolism in healthy adults, and on components of MetS in overweight and obese adults.

As we did not observe any significant clinical effects on glucose or lipid metabolism after the low dose levels used in this PhD project, future studies should preferably investigate the effect of supplementing with higher protein doses. The presence of peptide sequences with known bioactive sequences were not analysed in the protein hydrolysate used in the current project. If the hydrolysate is used in further studies, an analysis of peptide sequences with potential metabolic effects, such as glucose- or lipid lowering effects, should be conducted.

In the current project, cuttings and trimmings from the cod fillet production was used to produce the hydrolysate. It is of interest to further investigate if also other parts of residual materials retained after processing of fish, such as head, skin, entrail and backbone, have any beneficial metabolic effects and can be used for human consumption. Overall, increasing the utilization of residual materials from the fishing industry will contribute to increased profits for the fisheries and aquaculture, reduce waste and contribute to a sustainable fishing industry, ultimately contribute towards the achievement of SGDs within 2030.

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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

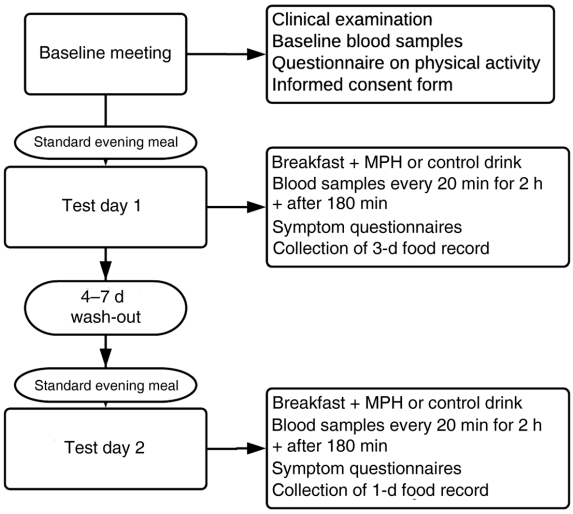

Fig. 1. Flowchart depicting the inclusion process for the study evaluating the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism in healthy individuals aged 40–65 years. Participants were recruited through advertisements on the Internet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

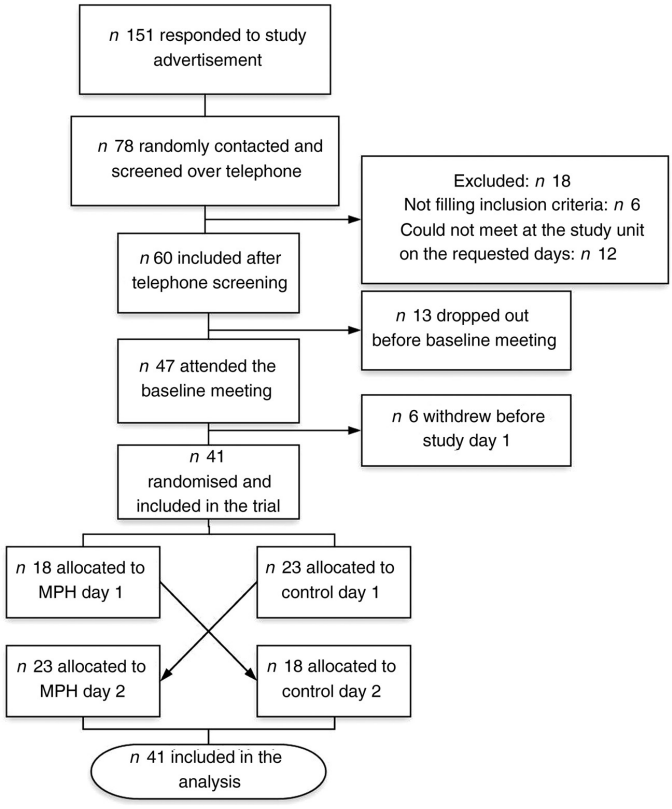

Fig. 2. Study protocol for the evaluation of the effect of a marine protein hydrolysate (MPH) from Atlantic cod (*Gadus morhua*) on postprandial glucose metabolism. We included forty-one healthy subjects (age range 40–64 years).

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 ($\exp^{(\log \text{ mean})}$).
* 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.

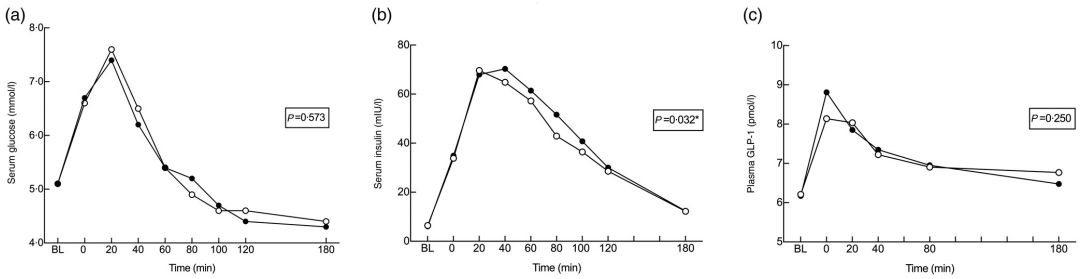

Fig. 3. Metabolic response for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) concentrations after intake of a standardised breakfast meal supplemented with a drink containing either marine protein hydrolysate (MPH; –○–) or control (casein; –●–). Results are presented 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

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II

JNS
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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

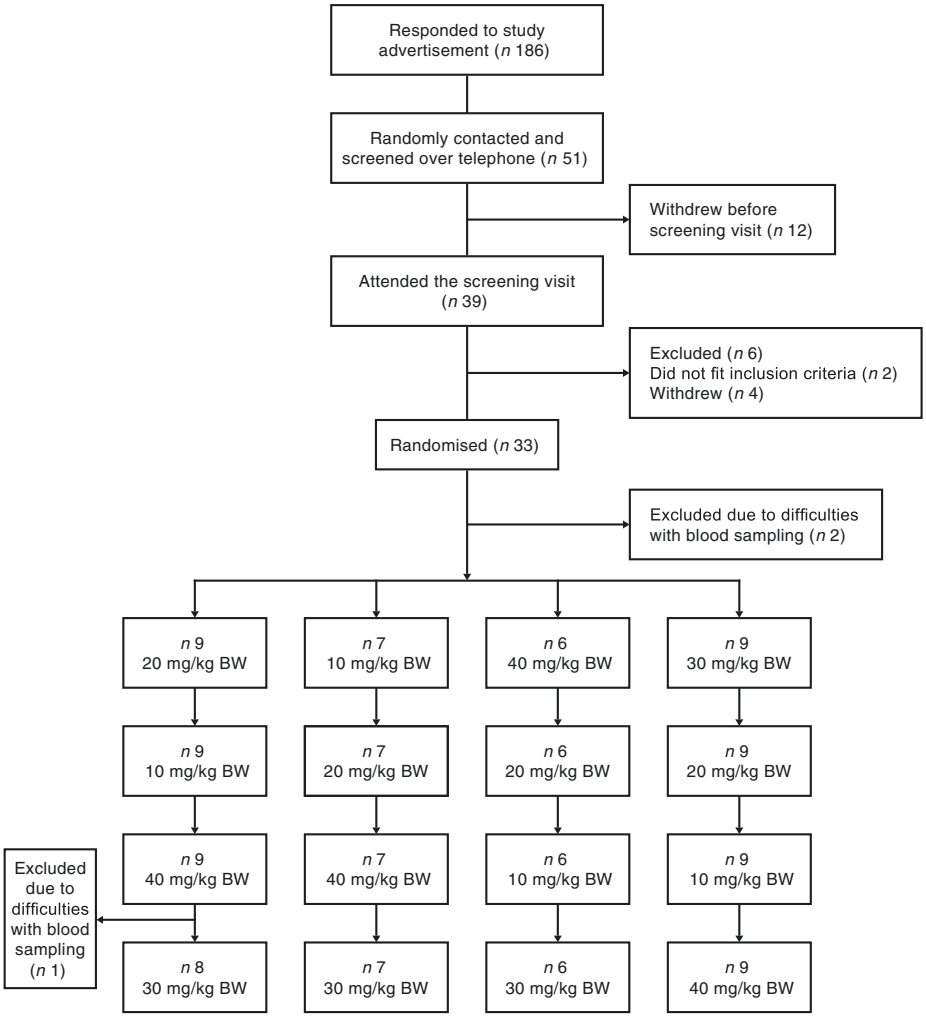

Fig. 1. Flowchart depicting the inclusion and randomisation process. BW, body weight.

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

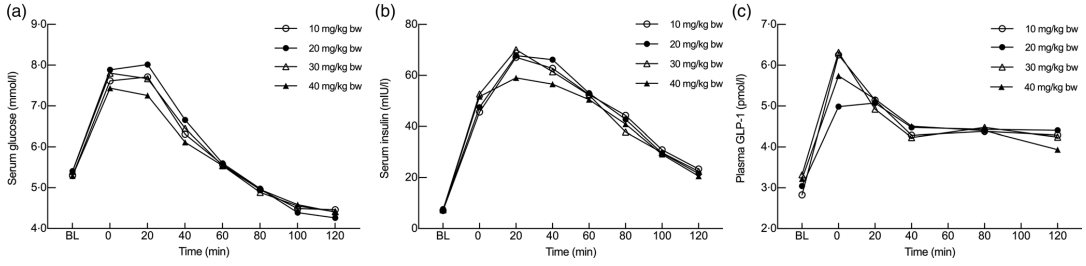

Fig. 2. Metabolic responses for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) concentrations after intake of a standardised breakfast meal and the last dosage of the cod protein hydrolysate (CPH). Dose levels were 10, 20, 30 and 40 mg/kg body weight (BW). Results 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.

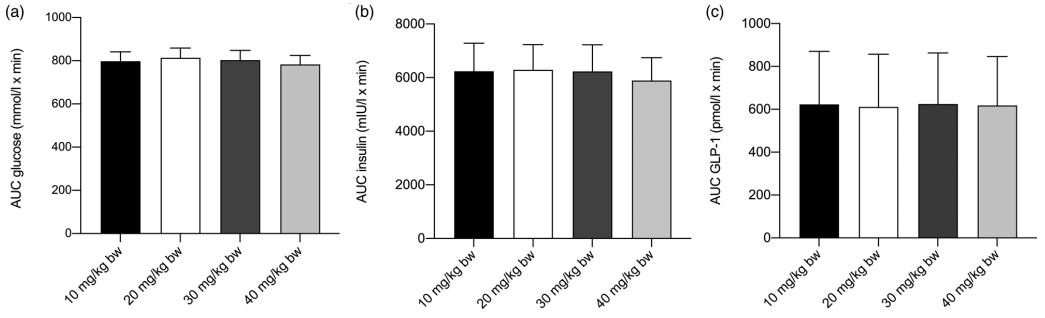

Fig. 3. Bar chart depicting the total AUC for serum glucose (a), serum insulin (b) and plasma glucagon-like peptide 1 (GLP-1) (c) after intake of a standardised breakfast meal and the last dosage of cod protein hydrolysate (CPH) in the dose level. Dose levels were 10, 20, 30 and 40 mg/kg body weight (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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III

nutrients

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Article

Supplementation with Low Doses of a Cod Protein Hydrolysate on Glucose Regulation and Lipid Metabolism in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study

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Abstract: The risk of cardiovascular diseases and type 2 diabetes mellitus are increased in subjects with metabolic syndrome (MetS), and hydrolyzed fish protein may have favorable effects on metabolic health. Here, we investigated the effect of 8 weeks supplementation with 4 g of cod protein hydrolysate (CPH) on glucose metabolism, lipid profile and body composition in individuals with MetS in a double-blind, randomized intervention study with a parallel-group design. Subjects received a daily supplement of CPH (n = 15) or placebo (n = 15). Primary outcomes were serum fasting and postprandial glucose levels. Secondary outcomes were fasting and postprandial insulin and glucagon-like peptide 1 (GLP-1), fasting lipid concentrations and body composition. No difference was observed between CPH and placebo for insulin, glucose or GLP-1 after 8 weeks intervention. Fasting triacylglycerol decreased in both the CPH group and placebo group, with no change between groups. Fasting total cholesterol and low-density lipoprotein cholesterol decreased significantly within both groups from baseline to study end, but no difference was observed between the two groups. In conclusion, supplementing with a low dose of CPH in subjects with MetS for 8 weeks had no effect on fasting or postprandial levels of insulin, glucose or GLP-1, lipid profile or body composition.

Keywords: metabolic syndrome; cod protein hydrolysate; glucose metabolism; lipid metabolism; obesity

1. Introduction

Hyperglycemia, hypertension, dyslipidemia and abdominal obesity form a cluster of interconnected metabolic abnormalities commonly known as the metabolic syndrome (MetS), which increases the risk of type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVDs) [1–3]. The prevalence of MetS varies depending on the definition used and the population studied, but it

is estimated that between 20 and 30% of the adult population in most countries meet the diagnostic criteria for MetS [4]. Currently, the first-line therapy for MetS is education on lifestyle changes including physical activity and weight reduction, and improvement of risk factors closely linked to MetS [3]. The syndrome has significant negative impact on public health, and the rate of MetS is expected to continue to rise in adults and future generations unless we find effective strategies to prevent and reverse this development [4]. It is of interest to find ways to prevent and alleviate MetS, beyond the currently used strategies.

Based on associations seen between fish consumption and increased levels of high-density lipoprotein cholesterol (HDL-C) and reduced levels of triacylglycerols (TAG), increased consumption of fish may improve metabolic health and prevent development of MetS [5–8]. The nutrients in fish, such as iodine, vitamin D, taurine, long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) and high-quality protein, may all contribute to the positive health effects of fish consumption [9]. There is also emerging evidence that proteins from fish contain bioactive peptides and may potentially modulate physiological processes in the human body, and contribute with a number of effects beyond their nutritional value as a source of energy and amino acids [10,11]. Bioactive peptides are released naturally by gastric digestion, produced by fermentation in the gut or through hydrolyzed protein added to the diet [12]. Animal and human studies suggest that hydrolyzed fish proteins given in low doses may have beneficial effects on lipid metabolism [13–15], postprandial glucose [14] and insulin regulation [16], as well as body composition and appetite [17,18]. Similar indications are observed in intervention studies with healthy overweight and obese adults given low doses (between 2.5 and 8 g) of supplements with unhydrolyzed cod protein [19–21].

We have previously investigated supplementation with low doses of a cod protein hydrolysate (CPH) on glucose metabolism and appetite in healthy adults [16,22], as well as supplementation for 6 weeks on inflammation and gastrointestinal health in patients with irritable bowel syndrome [23]. It is of interest to further evaluate the possible effects of low doses of CPH in a group of participants with metabolic abnormalities, such as subjects with MetS, over a longer period. Therefore, we aimed to investigate whether supplementation with low doses of 4 g of CPH per day for 8 weeks would have an effect on postprandial glucose metabolism and the appetite hormone glucagon-like peptide 1 (GLP-1), lipid profile and body composition in subjects with MetS. We hypothesized that the small peptides present in the CPH supplement would serve as rapidly absorbed bioactive peptides and lead to beneficial changes in glucose metabolism and an overall healthier metabolic profile.

2. Materials and Methods

2.1. Study Design

This study was as a multicenter, double-blinded, randomized, intervention with a parallel-group design. Participants with MetS received a daily supplement of 4 g of CPH (active ingredient) or placebo (no active ingredient) for 8 weeks. The primary outcome was fasting and postprandial glucose levels. Secondary outcomes were other metabolic and clinical parameters of the metabolic syndrome (waist circumference, fasting TAG and HDL-C), as well as fasting and postprandial insulin and GLP-1 levels, total cholesterol (total-C), fasting low-density lipoprotein cholesterol (LDL-C), and body composition.

All subjects gave their written informed consent for inclusion before participation in this study. This study was conducted in accordance with 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 (2018/2163). This study is registered at clinicaltrials.gov (NCT03807752).

2.2. Participants and Study Setting

Between March and September 2019, we recruited participants to this study through advertisement on the internal and external websites, on notice boards at Haukeland University Hospital (HUH), Bergen, and Ålesund Hospital, Ålesund, and at general practitioners in Bergen and the surrounding area.

The criteria for inclusion were age between 40 and 70 years, body mass index (BMI) between 27 and 35 kg/m^2 and the presence of MetS. Criteria for exclusion were chronic diseases or medication that were likely to interfere with the evaluation of the study endpoints (e.g., T2DM, medications known to affect glucose and lipid metabolism), allergy or intolerance to fish and/or shellfish, excessive alcohol consumption and/or drug as assessed by physician, acute infections or unwillingness to comply with the study requirements. As blood pressure was not an outcome measure, we allowed participants using certain types of blood pressure medications, not known to clinically affect glucose metabolism, to take part in this study. This included diuretics, calcium-channel blockers and agents acting on the renin-angiotensin system. Participants using beta-blocking agents or peripheral vasodilators were excluded. Four weeks prior to starting the intervention and during this study, the participants had to stop using any nutritional supplements with n-3 PUFAs. No changes in food consumption or level of physical activity were allowed. Lastly, the participants had to remain at a stable weight for the last three months and not be involved in any weight-loss programs prior to or during the intervention.

2.3. Definition of Metabolic Syndrome

The Joint Interim Statement was used to define MetS, in which the presence of any three of five risk factors qualifies for a diagnosis of MetS: elevated fasting glucose, s-TAG, reduced HDL-C, increased waist circumference (WC) or elevated blood pressure [1]. Furthermore, we used the International Diabetes Federation cut-off points for central obesity (WC ≥ 80 cm in women; ≥94 cm in men) [24]. The cut-off points for the other components were as follows: s-glucose ≥ 5.5 mmol/L, s-TAG ≥ 1.7 mmol/L, s-HDL-C < 1.0 mmol/L in men and <1.3 mmol/L in women, systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg [1].

2.4. Study Visits

All possible participants responding through the online recruitment form were pre-screened by telephone. Based on this, we invited potential participants to a screening visit to evaluate the presence of MetS and eligibility in terms of inclusion and exclusion criteria. The screening visit included a clinical examination by a physician, a review of medical history, vital sign (blood pressure, heart rate), anthropometric measures (weight, height and waist circumference) and blood sampling. We measured height and weight to the nearest 0.1 cm or 0.1 kg using an electronic weight/height scale and used these parameters to calculate BMI (kg/m^2). Furthermore, at screening and end of study visit, we measured waist circumference (WC) according to WHO recommendations [25,26], i.e., locating the midpoint between the lower margin of the last palpable rib and the top of iliac crest, with arms relaxed at the side, at the end of a normal expiration and using a stretch-resistant measuring tape with constant tension.

On days of study visits, the participants came to the study center in the morning. After an overnight fast (i.e., after 9:00 pm the previous day, the participants could not eat/drink or use nicotine), all study procedures were performed. Fasting blood samples were taken, and anthropometric measures were performed. Body composition was measured by a bioelectrical impedance analysis (BIA) device at baseline and end of study visit (Body Composition Analyzer, BC-418 MA (model used in Ålesund), MC-180 MA (model used in Bergen), Tanita Corporation, Tokyo, Japan), following the manufacturers' guidelines—barefoot with light clothing and an empty bladder.

Following the baseline measurement, the participants consumed a standardized breakfast meal (test meal). It consisted of two slices of semi-coarse bread (80 g bread, 50% whole wheat), 20 g white cheese, 25 g strawberry jam, 10 g margarine and 1.5 dL orange juice, providing a total of 1840 kJ (440 kcal), 69 g carbohydrate, 13.3 g protein and 14.3 g fat. We calculated the energy and macronutrient content using "*Kostholdsplanleggeren*" (Norwegian Food Safety Authority and The Norwegian Directorate of Health, Oslo, Norway) [27]. The participants consumed the meal within 15 min. Blood was drawn from an antecubital vein in the fasting state (−20 min), at 0 min, i.e., immediately after the meal was finished, and thereafter at 20, 40, 60, 80, 100 and 120 min. To induce an

adequate blood glucose response, we calculated the required amount of energy and macronutrient in the test meal. We allowed free drinking of water, but did not serve coffee or tea.

The participants started the intervention on the day following the baseline visit. They opened the sealed bag with powder (active or placebo) and mixed well with 100 mL cold water. The participants consumed the supplement 10 min before breakfast every day for 8 weeks, except for the morning of the end of study visit due to required fast.

2.5. Test Material

Firmenich Bjørge Biomarin AS (Ålesund, Norway) manufactured the test material. The flavored white powder came pre-packed in cardboard boxes from Pharmatech AS (Fredrikstad, Norway), with 56 sealed plastic-coated aluminum bags per box. The boxes were marked with A or B, i.e., blinded for both participants and personnel involved in this study. The intervention material contained 4 g of hydrolyzed protein from cod (cuttings and trimmings) in addition to 5 g glucose hydrate (Cargill), 2 g maltodextrin, 0.025 g tastegram powder flavor, 0.7 g citric acid and 0.1 g lemongrass durarome taste. The placebo contained 6.5 g of maltodextrin, 0.2 g citric acid, and was otherwise identical to the active material. The active material could not be identified from the placebo according to flavor or appearance.

The cod protein hydrolysate was produced by enzymatic hydrolysis of fresh frozen meat (cuttings and trimmings) from Atlantic cod (*Gadus morhua*). A batch of 500 kg frozen raw material was grounded, transferred to an incubator and mixed with sweet water at a ratio of 1:1, followed by stirring at 80 rpm and heating to 55 °C. The enzyme preparation Protamex® (Novozymes AS, Copenhagen, Denmark) was then added, following incubation for 45 min, at 55 °C and pH 7.0. The preparation (incubate) was heated to 90 °C for 15 min to inactivate the enzyme. The enzyme-inactivated incubate was passed through a rotating sieve (Swenco, Sweden) to remove any bone fragments. A two-phase centrifugation (Alfa Laval AS, Denmark) was used to separate the peptide-containing water-soluble fraction (the hydrolysate) from the indigested residue. This was followed by ultrafiltration and dehydration of the soluble phase to a 50% dry matter concentrate, which was spray dried to a powder. The spray dried CPH powder contained 89% crude protein, 0% carbohydrate, <0.2% fat, 0% carbohydrate, <3.0% water, 0.1% sodium chloride, 1.7% sodium, 0.07% chloride and 10% ash. Of the total amino acid content in the hydrolysate, the free amino acids constituted 4.77%, and the ratio between essential amino acids and non-essential amino acids was 0.70. When analyzing the molecular weight (MW) of the hydrolysate, approximately 90% of the peptides present in the hydrolysate had a MW of 2000 Daltons (Da) or less, which corresponds to peptides consisting of 18 amino acids or less. Furthermore, approximately 75% had a MW of 1000 Da or below, corresponding to 10 amino acids or less, and 55% had a MW of 500 Da, corresponding to 5 amino acids or less. Approximately 25–30% of the hydrolysate was small dipeptides and free amino acids, with a MW of less than 200 Da. The composition of amino acids and taurine content of the spray dried CPH powder are given in a previous publication [16].

2.6. Estimation of Energy and Macronutrient Intake

To determine individual diet habits, the participants recorded food and drink intake in a three-day prospective food diary, including one weekend day, before the baseline visit and before the end of study visit. Energy and protein intake from the supplement was added to the end of study dietary records (CPH group: 44 kcal, 4 g protein; placebo group: 46.5 kcal, 0 g protein). We used dietary records to evaluate whether any changes were made in the participants diets during the intervention period and to record diet patterns. Calculations of energy and macronutrient intake were determined using "*Kostholdsplanleggeren*" [27].

2.7. Analyses of Blood Samples

Samples for safety purposes (albumin, prealbumin, leucocytes, thrombocytes, hemoglobin, sodium, potassium, alanine aminotransferase, alkaline phosphatase, creatinine and aspartate aminotransferase)

were taken at the screening and end of study visit. Glycated hemoglobin (HbA1c), total-C, TAG, LDL-C, HDL-C and fasting and postprandial serum insulin and glucose were measured at baseline and end of study visit. All tests were analyzed according to standard accredited methods at the routine hospital laboratories (Department of Medical Biochemistry and Pharmacology, HUH, and Department of Medical Biochemistry, Ålesund Hospital).

Samples for GLP-1 determination were collected in Vacuette® EDTA-K2 tubes, ref#454047 (Greiner Bio-One GmbH, Frickenhausen, Germany), with 20 µL dipeptidyl peptidase-4 inhibitor (DPP4-010; DRG Diagnostics, Marburg, Germany) added prior to sampling. Plasma for fasting and postprandial GLP-1 at baseline and end of study was obtained by centrifugation of EDTA blood at 1800× *g* and −4 °C for 10 min, within 20 min after blood sampling. Plasma GLP-1 was analyzed by ELISA method (GLP-1, Active form (High Sensitivity ELISA), Code No. 27700, (Immuno-Biological Laboratories Co., Ltd., IBL Japan)).

2.8. Randomization

To allocate the participants, we used a web-based data collection and randomization system developed and administered by the Norwegian University of Science and Technology, Trondheim, Norway. We used block randomization to create the random assignment order and stratified for center (Ålesund or Bergen). A person with no practical involvement in the trial coded the test materials. The participants, study investigator, and all other personnel involved in this study were blinded to group allocation. Study investigators were given the key of randomization after the trial was finished and the statistical analyses were carried out.

2.9. Statistical Analyses

We performed statistical analyses by IBM SPSS Statistics, Version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.4.1 (GraphPad Software, Inc., San Diego, CA, USA. The latter was used for graphical work. The data is presented as the mean ± SD, unless otherwise stated. Normality was evaluated by the Shapiro–Wilk test and histograms, and non-normally distributed data was log-transformed before using parametric statistical tests. We tested changes within groups from baseline to end of study by the paired sampled *t*-test, and changes between groups by the independent samples *t*-test. We examined group differences over time for continuous outcome (postprandial measurements of insulin, glucose and GLP-1) in a linear mixed-effects model with repeated measures. The trapezoid rule was used when calculating the area under the curve (AUC) using only end of study day, comparing the active and placebo group. Level of significance was set to $p < 0.05$. Two participants in the placebo group were excluded from the statistical analysis of lipid parameters (TAG, total-C, LDL-C and HDL-C), due to the use of lipid-lowering drugs (Simvastatin (Zocor), Atorvastatin).

To our knowledge, the possible effects of supplementation with a hydrolyzed cod protein have not been investigated previously in overweight and obese subjects with MetS. Due to a lack of similar studies, a power calculation was not performed. According to the protocol, we planned to recruit 60 participants in this study, with 30 participants in each group (a minimum of 20 in each group). This is a number similar to what has been previously reported in studies with supplementation of low doses of cod protein in humans [20,21]. We did not reach the target population due to difficulties with the recruitment of eligible participants. Participants were recruited between April and September 2019, but due to time constraints and limited resources, the inclusion of new participants had to stop in September.

3. Results

3.1. Participants

Of 58 participants attending the screening visit, 35 subjects had MetS and could be included in the trial. Four participants withdrew before randomization. Thirty participants completed the intervention according to study protocol and were included in the statistical analysis (Figure 1). At the screening visit, the groups were comparable (Table 1).

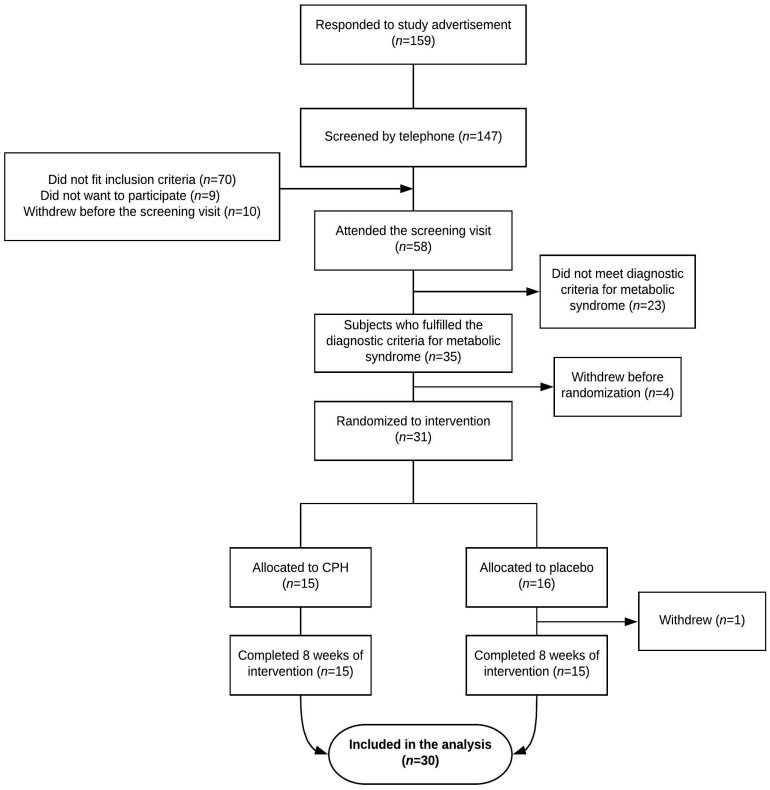

Figure 1. Overview of the participant flow during this study.

Table 1. Clinical and physical characteristics of subjects in the cod protein hydrolysate (CPH) group (n = 15) and the placebo group (n = 15) at screening.

Variables	CPH		Placebo		*p*-Value
	Mean	SD	Mean	SD	
Gender (female/male)	11/4		13/2		0.651
Age, years	52.8	6.26	53.4	6.83	0.804
Anthropometric measurements					
Body weight, kg	96.5	12.8	93.4	12.2	0.509
WC, cm	107.6	9.72	105.7	10.7	0.630
BMI, kg/m^2	32.7	2.24	32.4	3.25	0.751
Blood pressure (BP)					
Systolic BP, mmHg	136.9	15.9	138.5	15.1	0.756
Diastolic BP, mmHg	88.2	10.1	86.7	6.44	0.702
Glucose metabolism					
Glucose, mmol/L	5.73	0.75	5.63	0.79	0.704
HbA1c, mmol/mol	37.5	4.47	35.7	3.40	0.208
Lipid metabolism					
Total cholesterol, mmol/L	5.8	1.1	5.5	0.8	0.466
HDL cholesterol, mmol/L	1.3	0.3	1.4	0.2	0.493
LDL cholesterol, mmol/L	4.2	0.1	3.9	0.8	0.334
Triacylglycerol, mmol/L	2.10	0.7	2.05	0.6	0.870
Numbers using BP medications	5		9		-
Tobacco users	1		2		-

SD, standard deviation; WC, waist circumference; BMI, body mass index BP, blood pressure; BW, body weight; Hba1c, glycated hemoglobin. Results are presented as the mean ± SD. Groups were compared at baseline using independent samples t-test for continuous data and Fisher's Exact Test for categorical data.

3.2. Estimated Intake of Energy and Macronutrient

The estimated intake of energy and macronutrients calculated from the dietary records did not differ between the groups at baseline, and we did not observe any changes within or between groups during the course of this study (Table 2).

Table 2. Estimated energy and macronutrient intake in the cod protein hydrolysate (CPH) group (n = 15) and the placebo group (n = 15) at baseline and end of study (8 weeks).

Variable	Baseline		8 Weeks		p-Value [2]	p-Value [3]	p-Value [4]
	Mean	SD	Mean	SD			
Energy intake, kcal/day					0.668		0.726
CPH	1882	485	1777	466		0.177	
Placebo	1812	386	1746	410		0.406	
Protein, g/kg BW/day					0.922		0.815
CPH	0.91	0.22	0.85	0.24		0.357	
Placebo	0.91	0.29	0.87	0.23		0.569	
Fat, g/day					0.122		0.396
CPH	84.9	24.4	77.8	24.2		0.128	
Placebo	71.9	20.2	70.2	24.99		0.723	
Carbohydrate, g/day					0.323		0.488
CPH	193.2	46.0	191.7	51.3		0.848	
Placebo	211.1	51.5	198.4	49.3		0.207	
Basal metabolic rate[1], kcal					0.579		0.743
CPH	1760	334	1774	350		0.211	
Placebo	1696	296	1700	264		0.399	

SD, standard deviation; BW, body weight. [1] Derived from the bioimpedance analysis. [2] p-values comparing groups at baseline are based on independent samples t-test. [3] p-values within groups based on paired sample t-test. [4] p-values comparing change between groups are based on independent samples t-test. Data is based on the mean values from a three-day dietary records. Energy and protein content from the supplement was added to the end of study data—CPH group: 4 g protein, 44 kcal; placebo group: 0 g protein, 46.5 kcal. Results are presented as the mean ± SD.

3.3. Anthropometric Measurements

Body weight (kg), fat mass (kg, %), fat-free mass (kg), BMI (kg/m^2) or total body water (kg) did not differ within or between the groups (Table 3). Waist circumference increased within both the CPH group and the placebo group, but no differences were observed when comparing the two groups (Table 3).

3.4. Glucose Homeostasis

Adjusted for time and visit, the glucose levels were on average 0.55 mmol/L higher for CPH compared to placebo, but the linear mixed-effects model with repeated measures did not reveal any significant differences between the groups (95% CI: (−0.44, 1.53), p = 0.267). We observed no change in glucose levels from baseline visit to end of study visit in either of the two groups (overall change: −0.014 mmol/L, 95% CI: (−0.19, 0.16), p = 0.876) (Figure 2a). Similarly, no difference in insulin levels was observed between the two groups after 8 weeks intervention. The insulin levels were on average 0.63 mIU/L higher for CPH compared to placebo (95% CI: (−31.32, 32.58), p = 0.968), and we did not observe any changes in insulin levels from baseline visit to end of study in either of the groups (overall change: 0.57 mIU/L, 95% CI: (−4.79, 5.92), p = 0.836) (Figure 2c). Furthermore, no difference in fasting or postprandial GLP-1 levels was observed between the two groups. The GLP-1 levels were on average 0.83 pmol/L higher (back transformed estimate) for participants who received CPH compared to placebo, but this was not significantly different (95% CI: (0.61, 1.13), p = 0.221). We did not observe any changes in GLP-1 levels from baseline visit to end of study visit in either of the groups (overall change: 0.95 pmol/L, 95% CI: (0.90, 1.01), p = 0.079) (Figure 2e).

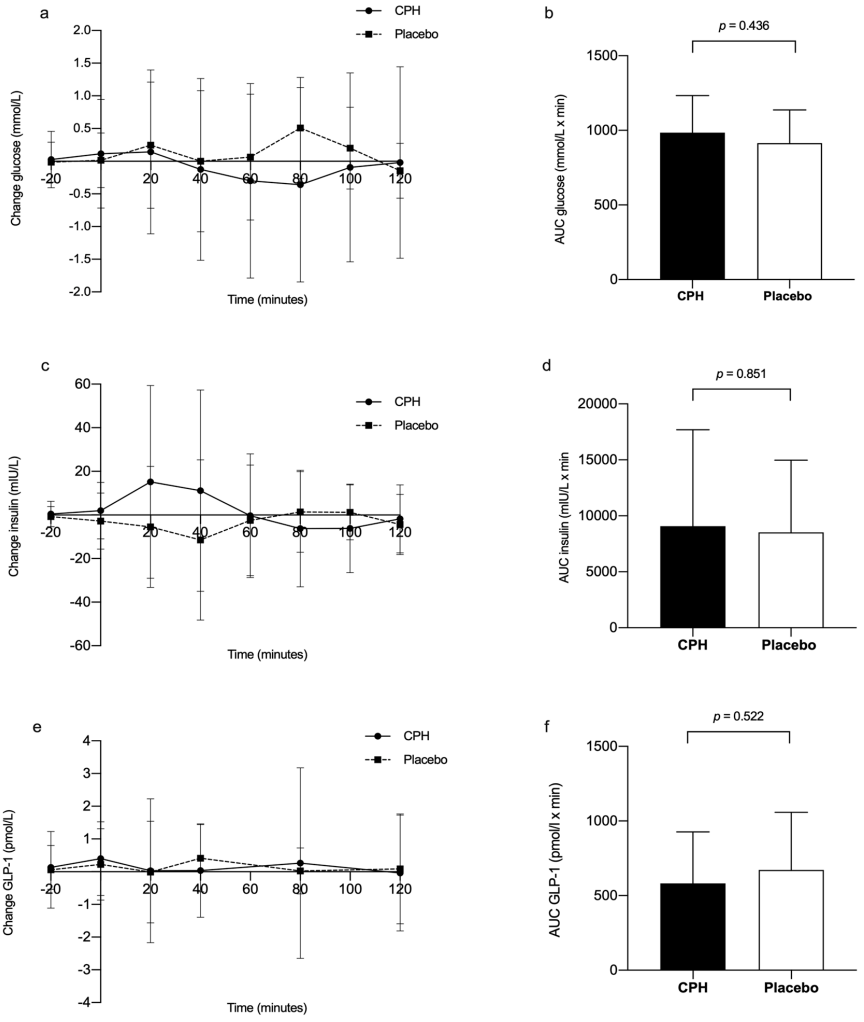

Figure 2. Glucose, insulin and glucagon-like peptide 1 (GLP-1) response in subjects with metabolic syndrome supplemented with cod protein hydrolysate (CPH) (n = 15) or placebo (n = 15) for 8 weeks. Change (8 weeks – baseline) in (**a**) serum glucose, (**c**) insulin and (**e**) plasma GLP-1 after a standardized breakfast meal, comparing the CPH group (solid line) with the placebo group (stippled line). The first postprandial blood samples (= 0 min) were taken 15 min after the test meal was served. For two individuals in the placebo group, only the fasting levels are included in the graphs. The area under the glucose curve (**b**, AUC glucose), insulin curve (**d**, AUC insulin) and GLP-1 curve (**f**, AUC GLP-1) for the CPH group (black bar) vs. the placebo group (white bar) at end of study. Values are presented as the mean ± SD.

Table 3. Anthropometric measurements and results from the bioimpedance analysis (BIA) in the cod protein hydrolysate (CPH) group (n = 15) and the placebo group (n = 15) at baseline and end of study (8 weeks).

Variable	Baseline		End of Study		p-Value[2]	p-Value[3]
	Mean	SD	Mean	SD		
Body weight, kg						0.557
CPH	96.02	13.6	96.14	13.8	0.715	
Placebo	93.15	12.7	92.93	12.2	0.694	
BMI, kg/m^2						0.603
CPH	32.55	2.43	32.59	2.51	0.741	
Placebo	32.27	3.45	32.21	3.49	0.692	
Waist circumference[1]						0.512
CPH	105.5	9.72	108.8	7.25	0.014	
Placebo	105.9	10.7	108.2	9.49	0.040	
Fat mass, %						0.897
CPH	39.91	6.79	39.50	7.12	0.211	
Placebo	40.21	5.37	39.75	5.47	0.105	
Fat mass, kg						0.834
CPH	38.16	7.81	37.82	8.12	0.319	
Placebo	37.46	7.05	37.03	7.46	0.163	
Fat-free mass, kg						0.816
CPH	57.81	11.54	58.33	12.01	0.221	
Placebo	55.71	10.08	55.92	9.02	0.301	
Total body water, kg						0.325
CPH	42.39	8.47	42.45	8.89	0.974	
Placebo	41.29	7.34	40.64	6.77	0.322	

SD, standard deviation; BMI, body mass index. [1] For waist circumference, the results are data measured at the screening visit, and presented for only n = 13 in the CPH group and n = 14 in the placebo group due to missing values. [2] P-values within groups are based on paired samples t-test. [3] P-values comparing change between groups are based on independent samples t-test.

We observed no significant interactions between group and visit (baseline vs. end of study), group and time, time and visit or between group, visit and time for insulin, glucose or GLP-1. The AUC calculated from the fasting through the postprandial test points did not reveal any significant differences in insulin, glucose or GLP-1 levels between the CPH group and placebo group after 8 weeks (Figure 2b, d and f, respectively).

At baseline, HbA1c was on average 37.5 ± 4.47 mmol/mol in the CPH group and 35.7 ± 3.40 mmol/mol in the placebo group, with no differences between groups (mean diff: 1.87, 95% CI: (−1.102, 4.835), p = 0.208). HbA1c did not change during the supplementation period within the CPH group (mean diff: 0.40 mmol/mol, 95% CI: (−1.15, 1.95), p = 0.589) or the placebo group (mean diff: 0.00, 95% CI: (−1.24, 1.24), p = 1), and there was no difference between the groups (mean difference: 0.40, 95% CI: (−1.49, 2.29), p = 0.669).

3.5. Lipid Parameters

At baseline, no differences between the groups were observed for fasting TAG, HDL-C, LDL-C, total-C or total-C: HDL-C ratio. Fasting total-C and LDL-C were significantly decreased within both groups after 8 weeks of intervention, with no differences between the groups (Figure 3). Fasting TAG was reduced within the CPH group (mean diff: −0.81 mmol/L (back transformed estimate), 95% CI: (0.694, 0.948), p = 0.012), but did not differ from placebo (Figure 3). We did not observe any changes within or between groups for for fasting HDL-C and total-C: HDL-C ratio during the course of the study (Figure 3).

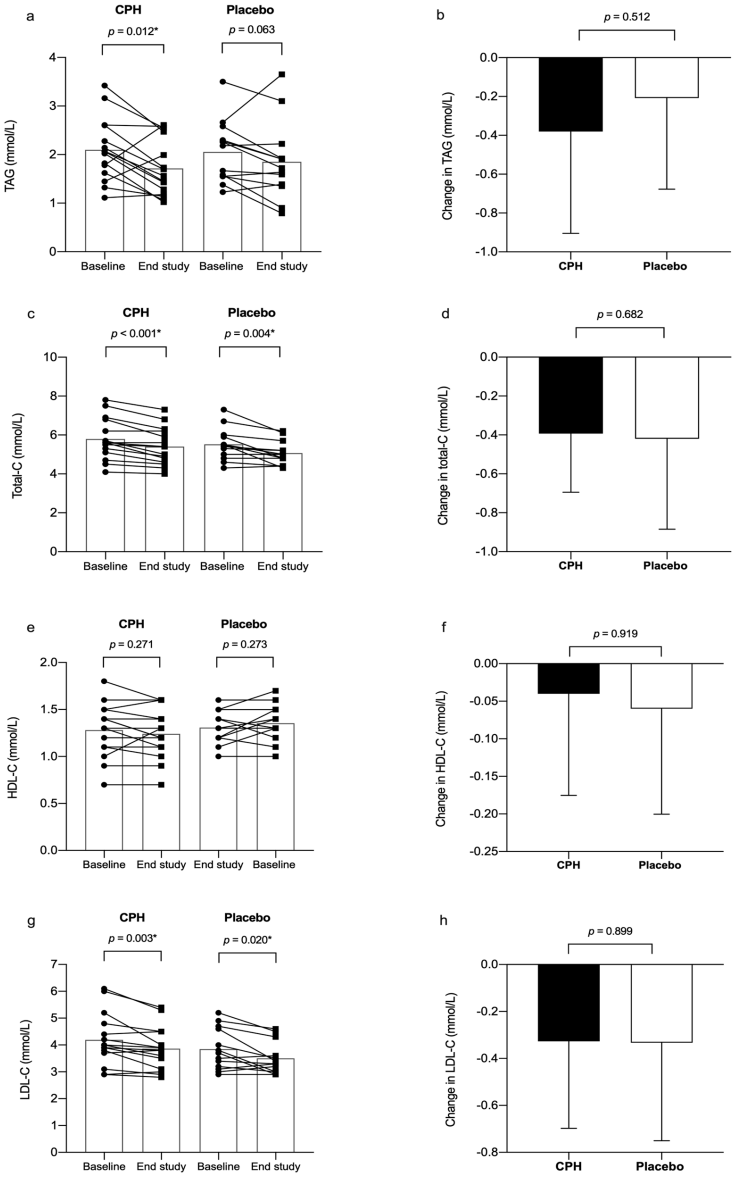

Figure 3. Fasting and change in serum levels of triacylglycerol (TAG) (**a**,**b**), total cholesterol (total-C) (**c**,**d**), high-density lipoprotein cholesterol (HDL-C) (**e**,**f**) and low-density lipoprotein cholesterol (LDL-C) (**g**,**h**) in subjects with MetS after 8 weeks intervention with cod protein hydrolysate (CPH) (n = 15) or placebo (n = 13). Two subjects from the placebo group were excluded from the statistical analyses of lipid parameter due to the use of lipid-lowering drugs. A (**c**,**e**,**g**), results are presented as individual graphs with fasting levels at baseline and end of study for each subject, with bars showing the mean in each group. p-values were calculated from a paired samples t-test. B (**d**,**f**,**h**), changes were calculated as individual end of study values minus baseline values for each of the indicators. Results are presented as the mean difference. p-values were calculated using the independent samples t-test. Significant p-values are marked with asterisk (*).

3.6. Adverse Effects

Biochemistry for safety purposes were all within normal range. Seven participants reported discomfort during the intervention period—four in the CPH group and three in the placebo group. One participant in the CPH group reported that the supplement tasted bad and caused retching. Two participants in the CPH group reported heartburn, and two reported nausea at the beginning of the intervention period, but this was transient. In the placebo group, one participant reported myalgia, one reported itchy rash in the face and one reported nausea, but all three participants were unsure whether these experiences were related to the intervention.

4. Discussion

The main aim of this study was to investigate whether daily supplementation with low doses of CPH for 8 weeks would have effect on fasting and postprandial glucose levels in participants with MetS. We hypothesized that supplementation with CPH would lead to beneficial changes in glucose metabolism and an overall healthier metabolic profile. We did not observe any significant effects of the supplement on the primary outcome measure (fasting and postprandial glucose levels) when compared to placebo. Furthermore, we found no effect on fasting or postprandial insulin or GLP-1 levels, lipid parameters or body composition after 8 weeks supplementation with CPH.

Previous human intervention studies have reported improvements in postprandial glucose and insulin levels after supplementation with fish protein [20,28], and similar findings have been observed in animal studies [14,29,30]. Furthermore, we have previously shown that the postprandial insulin concentration in serum was significantly lower in normal-weight adults given one single dose of CPH compared to control (unhydrolyzed casein), without an effect on glucose levels [31]. Considering that the subjects in the current trial had MetS, the lack of effect is surprising, but there could be several reasons for this. Firstly, we gave the participants a fixed dose of 4 g of CPH, whereas we used weight-adjusted doses of CPH in previous studies, to reduce the effect of body weight variations [16,31]. In the current trial, the participants in the CPH group had a body weight ranging from 77 to 133 kg, meaning variation from 52 to 30 mg/kg body weight of CPH per day, which could possibly affect the overall results. It was not feasible to weight-adjust the doses in the current trial, and we therefore chose to use a fixed dose similar to what has been used in other studies and based on results from a previous study conducted by our research group [20,21,31]. Secondly, the participants in the current trial only received a daily morning dose of CPH. Previous studies have suggested that distributing the doses throughout the day might lead to a more potent effect due to a constant flow of bioactive peptides in circulation [32,33].

The estimated average daily protein intake for participants in the CPH group was 85 g/day, and only 4.7% of the total daily protein intake came from the supplement. Therefore, we do not presume the protein intake as such to cause any effect on postprandial glucose regulation and lipid profile. We believe that an effect may be due to particular peptide sequences in the supplement. Bioactive peptide sequences have been identified in other fish protein hydrolysates [34]. We did not test for the presence of such sequences in this particular study, which would have strengthened the design.

Dietary proteins play a role in the regulation of lipid metabolism, and beyond the quantity of protein, the composition of amino acids and bioactive peptides are suggested to be of importance [35]. Associations between a high intake of lean fish and reduced levels of serum TAG [5,8], as well as increased levels of HDL-C [8], have been reported in previous cross-sectional studies. Beneficial effects have also been reported from intervention studies in animals and humans [36–38]. In the current trial, we observed a significant reduction in serum fasting TAG levels in the CPH group, with a decline of 18% from baseline to end of study. Considering that a weight reduction of 5–10% has been shown to cause a 20–30% reduction in TAG levels, whereas general improvement of nutrition-related practices can lead to a TAG-lowering effect of between 20 and 50% [39], a reduction of 18% in TAG levels after 8 weeks of supplementation with CPH, with no change in body weight or composition, is interesting. However, these changes did not differ from the placebo group, hence the results should be interpreted

with caution. In line with findings from previous intervention studies with low doses of cod protein in healthy, overweight adults [20] and normal-weight adults [33], we observed reduced levels of LDL-C within the CPH group. However, a reduction in LDL-C was also observed within the placebo group, and no differences were found when comparing the two groups. Overall, we did not observe any effects of 8 weeks CPH supplementation on lipid parameters.

Proteins are considered to be the most satiating of the macronutrients [40], and there are indications that fish protein, compared to other animal proteins like beef and chicken, have a greater effect on satiety [41]. The gut hormone, GLP-1, released in response to intake of food from the enteroendocrine L cells, is involved in appetite regulation and contributes to glycemic control by slowing gastric emptying, stimulating insulin secretion and suppressing the secretion of glucagon [42–44]. A previous study in overweight individuals found increased levels of GLP-1 after 3 months supplementation with 1.4 and 2.8 g of blue whiting hydrolysate, when compared to placebo (whey protein isolate) [17]. In the current trial, we did not see any effect of supplementation with 4 g of CPH for 8 weeks on fasting or postprandial GLP-1 levels. In the trial by Nobile et al. [17], all groups had a caloric restriction of −300 kcal per day, which may have influenced the result and might suggest that a reduction in calories in combination with supplementation of fish protein hydrolysate is a more effective approach than supplementation alone.

There are some limitations to the present study. Firstly, the lack of a power analysis and the small number of subjects per group might explain why we were not able to observe any effect of the intervention. Secondly, we did not reach the target population according to protocol, due to difficulties with recruiting eligible subjects and strict inclusion and exclusion criteria. We had to stop the inclusion period due to time constraints and limited resources, and the low number of subjects might have compromised this study. By choosing a cross-over design, we could have recruited fewer subjects and strengthened the design. This was not done in the current trial due to the long intervention period, which would require at least 4 weeks wash-out period in between the two experimental periods and the possibility of a high drop-out rate. Furthermore, participating in an intervention study may influence eating patterns and lead to underreporting of dietary intake. We instructed the participants to continue eating and activity level as normal during the course of this study, and we did not observe any changes in dietary intake or body weight. However, the estimated energy intake from the diet diaries were lower than anticipated according to weight. It should also be mentioned that body composition was assessed with BIA, which is suggested to be effective in healthy individuals and individuals with a stable water and electrolyte balance. The results should be interpreted with caution in individuals with BMI >34 kg/m^2 [45], and overestimating of fat-free mass has been observed when using BIA compared to dual-energy x-ray absorptiometry in obese subjects with BMI >35 kg/m^2 [46]. Since the average BMI in the current study was 32.5 kg/m^2, we assume that the results from the BIA analysis are reliable.

No previous publication, to our knowledge, has investigated supplementation with a low dose of a hydrolysate from cod in a population with MetS. To conclude, in this study, we showed that consumption of 4 g of a CPH daily for 8 weeks in individuals with MetS had no effect on fasting or postprandial glucose, insulin levels or GLP-1 levels, lipid profile or body composition. Studies in the future should further evaluate the effect of fish protein hydrolysate on lipid regulation, and preferably with a larger group of participants over a longer time period. Furthermore, the presence of potential bioactive peptide sequences with antidiabetic or lipid-lowering effects in the cod protein hydrolysate and the potential mechanism of effect should be explored further.

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IV

nutrients

MDPI

Article

The Effect of Supplementation with Low Doses of a Cod Protein Hydrolysate on Satiety Hormones and Inflammatory Biomarkers in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study

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Abstract: Metabolic syndrome (MetS) is characterised by metabolic abnormalities that increase the risk of developing type 2 diabetes mellitus and cardiovascular disease. Altered levels of circulating ghrelin, several adipokines and inflammatory markers secreted from adipose tissue, such as leptin, adiponectin, tumor necrosis factor alpha, are observed in overweight and obese individuals. We assessed the effect of supplementation with low doses of a cod protein hydrolysate (CPH) on fasting and postprandial levels of acylated ghrelin, as well as fasting levels of adiponectin, leptin and inflammatory markers in subjects with MetS. A multicentre, double-blinded, randomized controlled trial with a parallel group design was conducted. Subjects received a daily supplement of CPH (4 g protein, $n = 15$) or placebo (0 g protein, $n = 15$). We observed no effect on fasting or postprandial levels of acylated ghrelin, fasting levels of adiponectin ($p = 0.089$) or leptin ($p = 0.967$) after supplementation with CPH, compared to placebo. Overall, our study showed that 8 weeks supplementation with a low dose of CPH in subjects with MetS had no effect on satiety hormones or most of the inflammatory markers, but the levels of high-sensitivity C-reactive protein were statistically significantly different in the CPH-group compared to placebo group. The robustness and clinical relevance of these findings should be explored in future studies with a larger sample size.

Keywords: cod protein hydrolysate; satiety hormones; inflammatory markers; metabolic syndrome

1. Introduction

Metabolic syndrome (MetS) represents a cluster of metabolic abnormalities including abdominal obesity, hypertriglyceridemia, low levels of high-density cholesterol, hyperglycemia and hypertension, increasing the risk of cardiovascular disease and type 2 diabetes mellitus (T2DM) [1,2]. The pathogenesis of MetS is not fully understood, but a genetic predisposition combined with a sedentary lifestyle and

excessive caloric intake, are known to be important risk factors [3]. A positive energy balance over time leads to increased storage of fat in the adipocytes with expansion of adipose tissue, resulting in increased production of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) [3,4]. These cytokines and other signaling molecules secreted from the adipose tissue are involved in several physiological processes in the body, such as regulation of energy homeostasis, body fat accumulation and inflammation [5–7].

Inflammation is the immune system's response to an injurious stimulus, initiating a cascade of events promoting healing of the affected tissue [8]. Signaling pathways are activated as part of the inflammatory response, leading to the release of inflammatory mediators, including interleukin-1 beta (IL-1β), IL-6 and TNF-α [9,10]. Even though the acute inflammatory response is essential, the process may develop to a chronic inflammatory state, known to be involved in the development of several chronic diseases, such as diabetes [9]. Fish consumption has been linked to reduced levels of inflammatory mediators, such as IL-6 and TNF-α in rats [11,12] and healthy adults [13]. Lower levels of C-reactive protein (CRP), a marker of inflammation, have been observed in insulin-resistant subjects given a diet with lean fish (cod) for 4 weeks [14], with no effect observed when overweight individuals were given a low dose of cod protein supplementation [15]. The mechanism behind the possible anti-inflammatory effect of fish is largely unknown, but it has been suggested that the high taurine content in fish may have anti-inflammatory properties by suppressing IL-6 and TNF-α [12,16]. There are also indications from cell and animal studies, that bioactive peptides from lean fish have anti-inflammatory effects [9,17], with a need for further investigation in humans.

The secretion of IL-6, IL-8 and other cytokines are inhibited by adiponectin, a signaling molecule released from adipose tissue [4,18]. This molecule is known to enhance insulin sensitivity [4], and reduced levels have been reported in subjects with obesity and T2DM [5,19]. In contrast, increased levels of leptin, a hormone involved in regulation of food intake and appetite [7,20], have been reported in obese subjects [19]. Furthermore, consumption of fish and supplementation with *n*-3 polyunsaturated fatty acids (PUFAs) increase levels of adiponectin [19,21,22], with similar results reported after supplementation with cod protein in overweight and obese subjects [23]. For leptin, published data are conflicting [19,21,23,24]. As leptin and adiponectin seem to be altered in subjects with obesity and associated with factors of MetS, an effect on these adipokines by supplementation with fish protein, might be a possible preventive strategy for the development of MetS.

Ghrelin, a small peptide hormone secreted from the stomach, is an appetite-stimulating hormone with the opposite effects of leptin [25]. The levels of ghrelin increase before a meal and decrease postprandially [26], and is involved in regulation of appetite, energy balance and body weight [27]. Compounds that may inhibit the action of ghrelin and suppress appetite, may be beneficial for both prevention and treatment of components of MetS, such as obesity, impairments in lipid metabolism or glucose homeostasis [28]. We have previously reported the effect of low doses of a cod protein hydrolysate (CPH) on fasting and postprandial levels of acylated ghrelin in healthy adults [29]. We observed that a single dose of 20 mg/kg body weight of CPH did not affect postprandial levels of acylated ghrelin or sensations related to feeling of hunger, when compared to the control group [29].

Limited data exists on long-term supplementation of cod protein in a population with metabolic abnormalities, yet it is an important abnormality to study. We have previously reported on the effects on fasting and postprandial glucose metabolism, as well as lipid metabolism and body composition in subjects with MetS after supplementation with 4 g of CPH for 8 weeks [30]. In the present study, we aimed to investigate if daily supplementation with the same low dose of CPH for 8 weeks would influence circulating levels of ghrelin, adiponectin, leptin, high-sensitivity CRP (hs-CRP) and a selection of other inflammatory markers.

2. Materials and Methods

2.1. Study Design

We performed a multicenter, double-blinded, randomized parallel group trial with one daily dose of 4 g CPH or placebo for 8 weeks. Here, we report secondary outcomes; fasting and postprandial levels of acylated ghrelin and fasting levels of adiponectin, leptin and inflammatory markers. The study was conducted according to the Declaration of Helsinki and all procedures were approved by the Regional Committee for Medical and Health Research Ethics of Central Norway (2018/2163). Written informed consent was obtained from all subjects. The trial is registered at www.clinicaltrials.gov (NCT03807752).

2.2. Subjects and Study Setting

We recruited participants between March and September 2019 in the Bergen and Ålesund area (Norway) through an online recruitment questionnaire with advertisements on social media, at the participating hospitals and at general practitioners' offices. Inclusion criteria were diagnosis of MetS, body mass index (BMI) between 27–35 kg/m^2 and age between 40–70 years. Exclusion criteria were intolerance or allergy to fish and/or shellfish, chronic diseases or medication that were likely to interfere with the evaluation of study endpoints (e.g., T2DM, medications known to affect glucose metabolism), acute infections, abuse of alcohol or drugs (assessed by a physician) or unwillingness to comply with the study requirements. We included participants using calcium channel blockers (n = 3) agents acting on the renin-angiotensin system (i.e., ACE inhibitor n = 2, AII-receptor agonist n = 5, AII-receptor agonist/thiazide diuretic n = 3), since blood pressure was not an outcome. The participants were excluded if they had recently started with the current medication, if they had changed the dose level during the last 3 months or if it was changed during the study. Subjects using beta-blocking agents or peripheral vasodilators were excluded.

In this study, the Joint Interim Statement definition of MetS was used [1], where three abnormal findings out of five given risk factors qualifies for a diagnosis of MetS. The following risk factors and criteria were used: serum triglycerides ≥ 1.7 mmol/L, high-density lipoprotein cholesterol < 1.0 mmol/L in men and < 1.3 mmol/L in women, serum glucose ≥ 5.5 mmol/L, systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg [1]. For waist circumference (WC), we used the International Diabetes Federation cut-off points for central obesity: WC ≥ 94 cm in men, ≥ 80 cm in women [31].

2.3. Study Visits

After prescreening by telephone, we invited potential participants to a screening visit to assess eligibility, including inclusion and exclusion criteria. The screening visit included a clinical examination by a physician, review of medical history, measure of vital signs (blood pressure, heart rate), anthropometric measures (height, weight and WC) and blood sampling. We measured height (to the nearest 0.1 cm) and weight (to the nearest 0.1 kg) with an electronic scale (Seca 285, SECA GmbH, Hamburg, Germany). For measurements of WC, the WHO recommendation was followed [32,33]; the midpoint between the lower/inferior palpable rib and the top of iliac crest was located, the participants had arms relaxed at the side and the measurements were made at the end of normal expiration, using a measuring tape with constant tension. Changes in physical activity during the study period, as well as changes to food consumption were prohibited. The participants recorded their intake of food and drink in a three-day prospective food diary, before the baseline visit and end of study visit. Energy and protein intake from the supplement were added to the end of study dietary records (CPH group: 44 kcal, 4 g protein; placebo group: 46.5 kcal, 0 g protein). Details about the end of study energy intake is reported in a previous publication [30]. Calculations of energy and macronutrient intake were determined using "*Kostholdsplanleggeren*" (Norwegian Food Safety Authority, Norwegian Directorate of Health, Oslo) [34]. The participants had to stop the use of *n-3* PUFA-containing dietary supplements for four weeks prior to starting the study, and this was prohibited for the duration of the study.

After inclusion, the participants attended two identical study visits: the baseline visit and end of study visit (after 8 weeks of intervention). The participants came to the research facility between 08:00 AM–09:00 AM in a fasting state (no eating, drinking or use of any nicotine-containing substance after 09:00 PM the previous evening). When they arrived, fasting blood samples were taken followed by anthropometric measurements. Body composition was measured by a bioelectrical impedance analysis device (Body Composition Analyzer, Tanita Corporation, Tokyo, Japan, BC-418 MA (model used in Ålesund), or MC-180 MA (model used in Bergen)) according to the manufacturer's instructions. The participants then consumed a standardized breakfast meal (test meal) consisting of two slices of semi-dark bread (50% whole wheat, 80 g weight), 10 g margarine, 25 g strawberry jam and 20 g white cheese and 1.5 dL orange juice. The meal contained 1840 kJ (440 kcal), 69 g carbohydrate, 13.3 g protein, 14.3 g fat. The energy and macronutrient content of the test meal were calculated using *"Kostholdsplanleggeren"* [34]. The meal had to be consumed within 15 min and was followed by postprandial blood sampling. Blood was drawn from an antecubital vein prior to the test meal (−20 min), at 0 min (i.e., immediately after the meal was consumed), and thereafter at 20, 40, 60, 80, 100 and 120 min. No coffee or tea were served during test hours, but we allowed free drinking of water. We handed 8 weeks supply of the pre-packed test material (active or placebo) to the participants at the end of the baseline study visit. They started the intervention on the following day and took the supplement daily, 10 min before breakfast, for 8 weeks. Since the participants met fasting at both study visit, they did not take the study supplement at home before the end of study visit.

2.4. Test Material

The test material was manufactured by Firmenich Bjørge Biomarin AS (Ålesund, Norway), and delivered pre-packed in sealed plastic-coated aluminium bags. It was a lemon-flavored powder to be mixed with 100 mL cold water before ingestion. The powder bags with intervention material (CPH) contained 4 g of hydrolysed cod protein, 5 g glucose hydrate, 2 g maltodextrin, 0.025 g tastegram powder flavour, 0.1 g lemongrass durarome taste and 0.7 g citric acid. The placebo contained 6.5 g of maltodextrin, 0.2 g citric acid, and was otherwise identical to the intervention material. It was not possible to identify the CPH-material from the placebo, according to flavour or appearance.

The CPH was made by enzymatic hydrolysis of fresh frozen meat (cutting and trimmings) of Atlantic cod (*Gadus morhua)*, adding the enzyme preparation Protamex® (Novozymes AS, Copenhagen, Denmark) for 45 min, at 55 °C and pH 7.0. This was followed by inactivation of the enzyme, with heating to 90 °C for 15 min. The peptide containing water-soluble fraction (the hydrolysate) was separated from the indigested residue, followed by ultrafiltration and dehydration of the soluble phase to a 50% dry matter concentrate. This was spray-dried to a powder. The spray-dried CPH powder contained 89% crude protein and <0.2% fat, 0% carbohydrate, <3.0% water, 10% ash, 0.1% NaCl, 1.7% sodium and 0.07% chloride, by weight. Free amino acids accounted for 4.8% of the total amino acids in the hydrolysate, and the ratio between essential amino acids: non-essential amino acids was 0.70. Analysis of the molecular weight distribution, as well as the composition of amino acids and taurine content of the spray dried CPH powder is given in a previous publication [35].

2.5. Analyses of Blood Samples

Albumin, prealbumin, leucocytes, thrombocytes, hemoglobin, sodium, potassium, alanine aminotransferase, alkaline phosphatase, creatinine and aspartate aminotransferase, were analyzed at inclusion and end of study by standard accredited methods at Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital (HUH), and Department of Medical Biochemistry, Ålesund Hospital.

Serum for analyses of hs-CRP, adiponectin, leptin and inflammatory markers were 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. Hs-CRP was analyzed by standard accredited methods at the Department of Medical Biochemistry and Pharmacology, HUH. Serum adiponectin and

leptin were analyzed using Human Adiponectin High Sensitivity ELISA kit (Cat. No.: RD191023100, Biovendor, Brno, Czech Republic) and Human Leptin ELISA, Clinical Range kit (Cat. No: RD191001100, Biovendor) respectively. TNF-α and IL 1β, IL 6, and IL 8 were analyzed by the Cytokine human ultrasensitive magnetic 10-plex panel for Luminex™ platform (Cat.No: LHC6004M, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Leptin, adiponectin, as well as the inflammatory markers, were only measured and analyzed in the fasted stated.

Samples for ghrelin measurement were collected in Vacuette® EDTA Aprotinin tubes (Item No: 454261, Greiner Bio-One International GmbH, Kremsmünster, Austria), added 34 µL 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) Ready-made solution (Item No: SBR00015, Sigma-Aldrich, Saint-Louis, MO, USA), right after blood sampling. Plasma for fasting and postprandial ghrelin at baseline and end of study was obtained by centrifugation of EDTA blood at 1800× *g* at −4 °C for 10 min, within 20 min after blood sampling. Ghrelin levels were analyzed using the Ghrelin Acylated Human Easy Sampling ELISA (Cat.No: RA194062500R, Biovendor)

2.6. Randomization

To allocate the study participants, we used wCRF®, a randomization and data collection system developed by the Norwegian University of Science and Technology, Trondheim, Norway. The random assignment order was created using block randomization, and we stratified for center (Ålesund or Bergen). A person with no direct involvement in the study coded the test materials and the participants, as well as all study personnel involved in the study implementation and data handling, were blinded to group allocation.

2.7. Statistical Analyses

We performed statistical analyses using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.4.2 (GraphPad Software, Inc., San Diego, CA, USA). The Shapiro-Wilk test and histograms were used to evaluate normality. For data not following a normal distribution and not improved by log-transformation, we used non-parametric tests. The Wilcoxon's Signed Rank Test was used to investigate changes from baseline to end of study within groups, and the Independent Samples Mann Whitney U Test was used to compare changes (8 weeks—baseline) between the CPH and placebo group at end of study. These data (adiponectin, leptin, hs-CRP and inflammatory markers) are presented as median and interquartile range. A linear mixed-effects model with repeated measures was used to examine group differences over time for fasting and postprandial measurements of ghrelin. A Pearson's correlation coefficient analysis was used to examine relationship between fasting levels of acylated ghrelin at baseline and change in body weight (kg) and BMI (kg/m^2). The level of significance was set to $p < 0.05$. A power calculation was not done in the original study due to lack of data to base it upon [30]. Therefore, no estimation of sample size of the current measurements was done prior to the study. According to protocol, we planned to recruit 60 subjects in the study, which is a number similar to what have been reported in other supplementation studies with low doses of cod protein [15,36].

3. Results

3.1. Participant Characteristics

We screened a total of 147 participants for compliance with inclusion and exclusion criteria by telephone and invited 68 participants for a screening visit. Fifty-eight attended the screening visit and 30 participants were included and completed the intervention according to study protocol (Figure 1). Baseline characteristic are presented in Table 1.

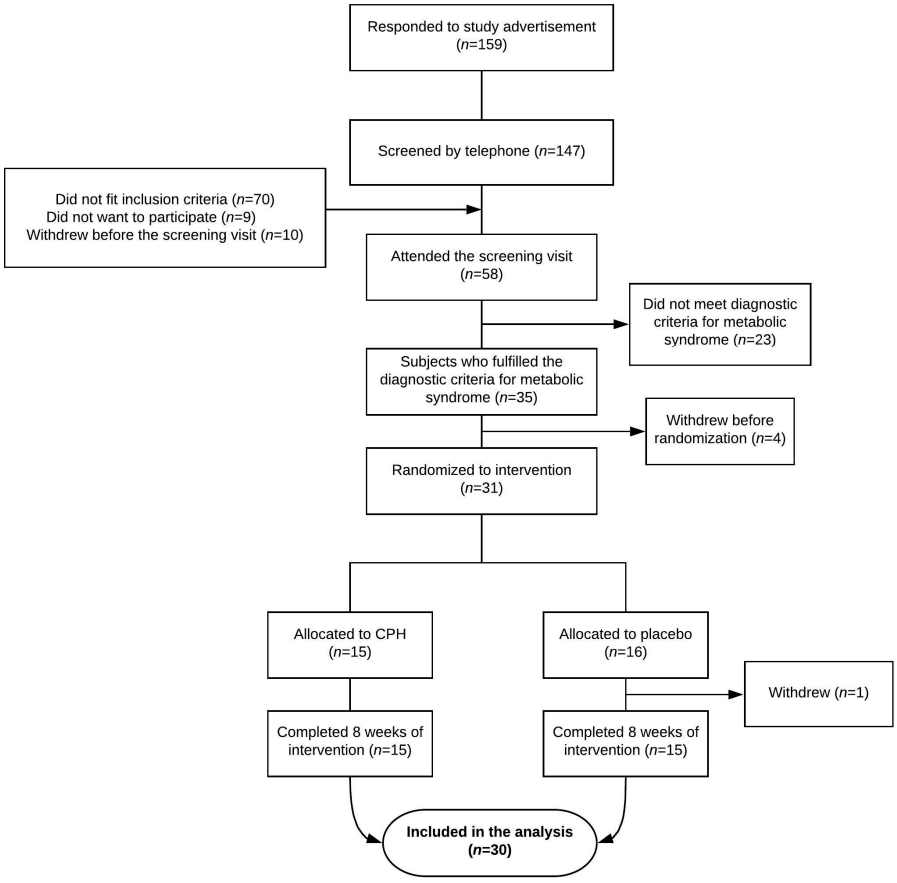

Figure 1. Participant flow during the study.

Table 1. Characteristics of the participants in the cod protein hydrolysate (CPH) group (n = 15) and the placebo group (n = 15) before intervention.

Variable	CPH		Placebo		*p*-Value
	Mean	SD	Mean	SD	
Gender, female/male	11/4		13/2		0.651
Age, years	52.8	6.26	53.4	6.83	0.804
Body weight, kg	96.5	12.8	93.4	12.2	0.509
BMI, kg/m^2	32.7	2.24	32.4	3.25	0.751
Waist circumference	107.6	9.72	105.7	10.7	0.630
Systolic BP, mmHg	136.9	15.9	138.5	15.1	0.756
Diastolic BP, mmHg	88.2	10.1	86.7	6.44	0.702
Energy intake, kcal	1882	485	1812	386	0.668
Protein intake, g/kg BW/day	0.9	0.2	0.9	0.3	0.992
Antihypertensive, *n*	5		9		–
Smokers, *n*	1		2		–

SD, standard deviation; BMI, body mass index; BP, blood pressure, BW; body weight. Results are presented as mean ± SD. Groups were compared at baseline using Independent Samples *t*-test.

3.2. Adiponectin and Leptin

No statistically significant differences between the groups were observed for adiponectin ($p = 0.806$) or leptin ($p = 0.367$) at baseline. At end of study, the fasting adiponectin concentration was significantly increased within the CPH group (baseline: 7.98 (5.68, 11.06) µg/mL, end study: 8.84 (6.06, 13.7) µg/mL), $p = 0.008$), with no changes observed within the placebo group (baseline: 7.89 µg/mL (7.06, 10.53), end of study: 7.82 (7.07, 11.90) µg/mL, $p = 0.910$) (Figure 2a). The median adiponectin change (8 weeks—baseline) in the CPH group was 0.56 (0.25, 1.71) µg/mL, and −0.12 (−0.56, 1.07) µg/mL in the placebo group. When comparing the change in fasting levels of adiponectin, no statistically significant difference between groups was observed ($p = 0.089$).

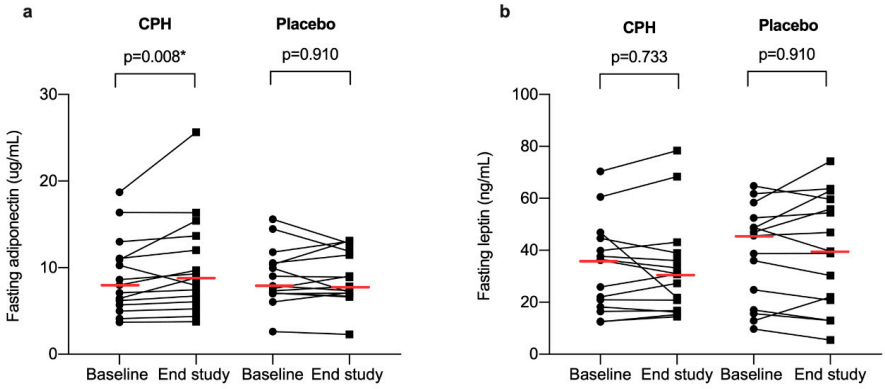

Figure 2. Fasting serum levels of adiponectin (**a**) and leptin (**b**) in participants with metabolic syndrome at baseline and after 8 weeks supplementation (end study) with cod protein hydrolysate (CPH) ($n = 15$) or placebo ($n = 15$). The red horizontal line shows the median levels. *p*-values within groups were calculated using the Wilcoxon's Signed Rank Test. Statistically significant *p*-values are marked with an asterisk (*).

No statistically significant differences in leptin levels within the CPH (baseline: 36.2 (18.2, 44.6) ng/mL, end study: 30.8 (16.8, 40.0) ng/mL, $p = 0.733$) or placebo group (baseline: 45.7 (17.1, 52.5) ng/mL end study: 39.6 (20.8, 59.6) ng/mL, $p = 0.910$) were observed from baseline to end of study (Figure 2b). The median leptin change in the CPH group was 0.30 (−3.27, 4.94) ng/mL, and 0.012 (−4.24, 8.98) ng/mL in the placebo group. When comparing the change in fasting levels of leptin, we did not observe any statistically significant difference between groups ($p = 0.967$).

3.3. Acylated Ghrelin Levels

At baseline, the levels of acylated ghrelin were 77.8 (196.2) pg/mL in the CPH group, and 24.9 (20.8) pg/mL in the placebo group. Adjusted for time and visit, the acylated ghrelin levels were on average 51.1 pg/mL higher for the CPH group compared to placebo, but the linear mixed effects model with repeated measures analysis did not reveal any statistically significant differences between the groups (95% CI: (−54.5, 157.0), $p = 0.330$). We observed no statistically significant change in acylated ghrelin levels from baseline to end of study visit in either of the groups (overall change: 0.03 pg/mL, 95% CI: (−1.50, 1.53), $p = 0.937$) (Figure 3). There were no significant interactions between group and visit (baseline vs. end of study, $p = 0.749$), group and time ($p = 0.693$), time and visit ($p = 0.794$) or between group, visit and time ($p = 0.853$) for acylated ghrelin.

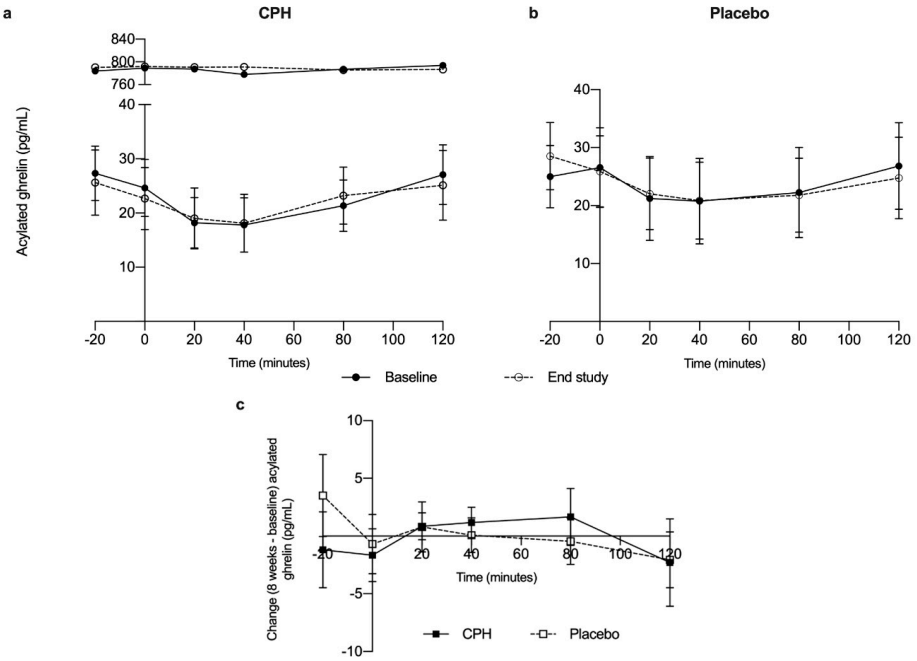

Figure 3. Fasting and postprandial acylated levels of ghrelin after a standardized breakfast meal at baseline (solid line) and end of study (dotted line) in participants that received supplementation with cod protein hydrolysate (CPH) (n = 15) (**a**) or placebo (n = 15) (**b**) for 8 weeks. In the CPH group, one individual had much higher levels of acylated ghrelin compared to the rest of the group and is shown as a separate segment on the graph. Graph (**c**) presents the change (calculated as 8 weeks—baseline) in acylated ghrelin during the intervention in the CPH group (solid line) compared to the placebo group (dotted line).

No correlations were observed between fasting concentration of acylated ghrelin and body weight (kg) (r = 0.075, p = 0.700) or BMI (r = 0.172, p = 0.372) for the whole group at baseline (results are presented for n = 29, one participant excluded from the correlation analysis due to high levels). Furthermore, no correlations were observed between changes in fasting acylated ghrelin levels and changes in body weight (kg) (r = −0.144, p = 0.457) or BMI (r = −0.146, p = 0.448).

3.4. Inflammatory Parameters

The values of hs-CRP and inflammatory markers in serum are shown in Table 2. The serum concentrations of the cytokines were low, but detectable.

No differences between the groups were observed for hs-CRP or any of the other inflammatory mediators at baseline. The concentrations of IL-1β, IL-6, IL-8 or TNF-α did not change during the course of the study and were not affected by supplementation with CPH (Table 2). After 8 weeks, the fasting level of hs-CRP was significantly higher within the CPH group, with no changes observed within the placebo group (Table 2). The median hs-CRP change in the CPH group was 0.1 (0.0, 2.0) mg/L, and −0.1 (−1.0, 0.55) mg/L in the placebo group. When comparing the change in fasting levels of hs-CRP, the distribution in the CPH group was significantly different from the placebo group (p = 0.029) (Table 2). Two participants in the placebo group were excluded from the statistical analysis of hs-CRP due to the use of lipid-lowering drugs (simvastatin, atorvastatin), because these are known to affect the levels of hs-CRP [37]. The statistical significance of results did not change if they were included in the statistical analysis of hs-CRP.

Table 2. The concentration of inflammatory markers in serum samples collected before and after 8 weeks supplementation with cod protein hydrolysate (CPH) (n = 15) or placebo (n = 15).

	Baseline		8 Weeks		p-Value [1]	p-Value [2]
	Median	**25th, 75th Percentile**	**Median**	**25th, 75th Percentile**		
Hs-CRP, mg/L						0.029 *
CPH	4.0	1.0, 4.0	4.0	2.0, 6.0	0.021 *	
Placebo	3.0	1.5, 7.0	3.0	2.0, 7.0	0.389	
IL-1β, pg/mL						0.567
CPH	0.13	0.13, 0.41	0.13	0.13, 0.41	0.574	
Placebo	0.13	0.13, 0.41	0.13	0.13, 0.41	0.589	
IL-6, pg/mL						0.935
CPH	1.04	0.52, 1.77	0.90	0.74, 1.48	0.394	
Placebo	1.19	0.75, 1.34	1.04	0.59, 1.34	0.396	
IL-8, pg/mL						0.174
CPH	15.8	11.9, 20.3	17.6	14.0, 22.5	0.096	
Placebo	18.1	15.1, 26.9	16.7	12.2, 23.0	0.281	
TNF-α, pg/mL						0.935
CPH	0.57	0.22, 0.93	0.93	0.22, 0.93	0.573	
Placebo	0.22	0.11, 0.93	0.57	0.22, 0.93	0.280	

Hs-CRP; high-sensitivity C-reactive protein; IL, interleukin; TNF-α, tumor necrosis factor alpha. The data are presented ad median and interquartile range. (1) p-values within groups are based on Wilcoxon's Signed Rank Test. (2) p-values between groups are based on Mann Whitney U Test. Statistically significant p-values are marked with an asterisk (*).

3.5. Adverse Effects

The blood tests taken for safety purposes were all within normal range. Seven subjects reported discomfort during the intervention period: four in the CPH group and three in the placebo group. In the CPH group, two subjects reported heartburn, two reported nausea at the beginning of the intervention period, and one reported that the supplement tasted bad and caused retching. In the placebo group, one reported itchy rash in the face, one reported nausea and one reported myalgia, but all three subjects were unsure if the symptoms were related to the intervention.

4. Discussion

The main objective of the present study was to investigate whether daily supplementation with low doses of CPH for 8 weeks would have an effect on circulating levels of ghrelin, adiponectin, leptin and different inflammatory markers in subjects with MetS. We hypothesized that 8 weeks supplementation with CPH would lead to a beneficial effect on circulating leptin levels and reduced inflammatory markers, as well as increased circulating levels of adiponectin and a suppressing effect on postprandial levels of ghrelin. These possibly beneficial effects were hypothesized to occur due to the presences of small peptides, mainly di- and tripeptides, suggested to be absorbed rapidly from the gastrointestinal tract and possibly influencing pathways involved in regulation of appetite and inflammation. Here, we show that 4 g of CPH for 8 weeks did not influence fasting and postprandial concentrations of acylated ghrelin or fasting levels of adiponectin, leptin or the inflammatory markers, when compared to placebo in individuals with MetS.

A daily supplementation with 4 g of CPH was sufficient to increase fasting serum adiponectin levels within the CPH group, with no change in serum levels of leptin in circulation. These results are in agreement with a previous study in healthy overweight and obese subjects receiving 2.5 g of cod protein (not hydrolyzed) for 8 weeks [23]. Observing the individual levels of adiponectin (Figure 2a), it is apparent that there are individual variations. Since the increased levels of adiponectin only were found within the CPH group and not when comparing the groups after intervention, the results should be interpreted with caution and should be explored in further studies. Still, these findings are of

interest and suggests that hydrolyzed protein from lean fish may have beneficial effects on adiponectin concentration. We hypothesize that an effect may be mediated by rapidly absorbed di- and tripeptides present in the hydrolyzed supplement, containing bioactive sequences affecting metabolic pathways in the cells and thereby increasing the levels of adiponectin. Compared to the recommended daily protein intake in healthy individuals (e.g., 0.8–1.5 g protein/kg body weight/day) [38], the amount of protein in the supplement is very low, and it is plausible that it is not the increased protein content per se that is responsible for the possible metabolic effect. Bioactive peptide sequences with effect on glucose metabolism, blood pressure and lipids have been identified in other fish protein hydrolysates [39]. We have not tested for the presences of these specific known bioactive sequences in our hydrolysate, which is a limitation of the study, and the possible mechanism of action is therefore only a hypothesis.

No effects on fasting or postprandial levels of acylated ghrelin were observed after supplementation with CPH, which is in line with what we observed after giving one single dose of CPH to normal weight adults [29]. In contrast to our previous study, we used a higher dose, over a longer period, and a population with metabolic disturbances in the current study. We still did not observe any effects on circulating acylated ghrelin. In the previous study we assessed self-reported feeling of satiety and hunger [29]. An assessment of appetite was not included, which would have been an improvement of the study design seen in relation to ghrelin. By including validated questionnaires for reporting appetite and a free eating lunch at each study visit, we would be able to calculate energy intake and assess whether supplementation with CPH for 8 weeks led to lower energy intake, and thereby suggesting a suppressing effect on appetite.

Fish has been proposed to have anti-inflammatory properties, and reduced levels of CRP have been linked to fish consumption [13]. A diet with cod protein reduced the levels of hs-CRP in insulin-resistant subjects [14], whereas others have reported of no effect on CRP levels or other inflammatory markers after a high intake of cod in normal-weight [40] or overweight subjects [41]. No effect on CRP levels was observed in an intervention study with overweight and obese subjects supplemented with similar amount of cod protein (intact protein) as used in the current trial [15]. We observed higher levels of hs-CRP after supplementation with CPH for 8 weeks, when compared to placebo, but did not observe any changes in other inflammatory markers. An increased level of CRP was reported in elderly subjects living in a nursing home setting given 5.2 g of fish protein (blue whiting) for 6 weeks (compared to placebo) [42], however, this group is not comparable to our study population. Overall, the higher levels of hs-CRP in the CPH group is difficult to interpret. It is possible that the small sample size might have influenced the results, or that some individuals may have had some on-going low-grade inflammation without disclosing a problem. When comparing the overall fasting levels of hs-CRP in both groups in our study with a previous study by Delongui et al. [43], our levels are similar to the levels reported in obese subjects and higher compared to normal weight subjects, emphasizing how BMI may affect CRP levels [43].

There are certain strengths and limitations to the study. Firstly, the randomized, double-blinded design is a strength. A cross-over design would potentially have been an even better design, with each subject serving as his/her own control and allowing us to recruit fewer subjects without compromising the strength of the study [44]. Since this would have resulted in a long intervention period and possibly higher dropout rate, we chose to conduct the current study as a parallel group study. Secondly, adjusting the CPH dosage to the body weight of each participant could have further strengthened the design, as it would reduce the effect of variation in body weight. It was not practically feasible, and we chose to use a dose similar to what has previously been effective [15,36,45]. Thirdly, the lack of a power analysis is a weakness when interpreting the data, but there was no relevant data to base such calculation upon. As we had difficulties recruiting, we did not reach our target of 60 subjects (with 30 individuals in each treatment arm), which might have further affected the results. In particular we had difficulties recruiting males and a predominance of female participants were included in the study. Due to limited resources and time constraint, the inclusion of new participants was stopped in September 2019. It cannot be ruled out that our results might have changed in a larger study, so replication from

future studies are necessary to examine the robustness of our findings, in particular related to fasting levels of serum adiponectin and hs-CRP.

5. Conclusions

To conclude, our study showed that a daily supplement of 4 g of CPH for 8 weeks was not sufficient to affect fasting or postprandial levels of ghrelin, or fasting levels of adiponectin, leptin or inflammatory markers in overweight and obese subjects with MetS.

Author Contributions: Conceptualization, C.J., T.H., J.G.H, G.A.L. and D.A.L.H..; Data curation, C.J.; Formal analysis, C.J. and I.B.; Investigation, C.J. and H.F.D.; Project administration, C.J. and D.A.H.L.; Supervision, T.H., J.G.H, G.A.L. and D.A.L.H.; Validation, I.B. and D.A.L.H.; Visualization, C.J.; Writing—original draft preparation, C.J.; Writing—review and editing, C.J., H.F.D., T.H., J.G.H., I.B., G.A.L. and D.A.L.H. All authors have read and agreed to the published version of the manuscript.

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

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V

Errata

Page 04 Incorrect abbreviation: The abbreviation “HMG: 3-hydroxy-3-methylglutaryl” is not used in the thesis and has been removed from List of Abbreviations.

Page 13 Incorrect abbreviation: “The National Cholesterol Education Program’s Adult Treatment Panel III” is abbreviated to ATP III. This is changed to NCEP ATP III. This has also been corrected in List of Abbreviations Page 4.

Page 13 “(...), and reduced high density HDL-C)” changed to “(...), and reduced HDL-C”.

Page 14 Table 1: An explanation for SPB (systolic blood pressure) and DPB (diastolic blood pressure) are missing in the table footer, and have been included.

Page 23 Missing word: “(…), with significant effects on lipid markers in other studies” - changed to “(…), with no significant effects on lipid markers in other studies”

Page 25 Misspelling: “(…) defined as “*a person’s weight in kilogram (kg) divided by the square of hens heights in meters (kg/m^2)*” changed to *“*(…) defined as “*person’s weight in kilogram divided by the square of his height in meters (kg/m^2)”*

Paper I: Page 5, Line 7, first paragraph “To EDTA blood sampling tubes were added (…)” – should be “The EDTA blood sampling tubes were added (…)”

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