Effect of prebiotic fibers on subjective and objective appetite markers in people with type 2 diabetes

A randomized placebo-controlled crossover trial

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Master thesis

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Abstract

Background: Type 2 diabetes mellitus (T2DM) is an increasing global problem, with high numbers of people living with the disease. Prebiotic fibers are shown to reduce hunger and increase satiety in healthy and obese people, through mechanisms involving the gut microbiota, gut hormones and the brain. However, the effect of prebiotic fiber supplements on appetite has not been studied in people with T2DM.

Purpose: The purpose of this study was to assess the effect of prebiotic fiber supplement on subjective and objective appetite markers in people with T2DM.

Materials and methods: Twenty-five participants with T2DM completed two 6-weeks periods with prebiotics (inulin and fructo-oligosaccharides) and placebo (maltodextrin) divided by a 4-weeks washout period in a double-blind, placebo-controlled, crossover trial. Subjective feeling of appetite, food intake and appetite related hormones (acyl ghrelin, active GLP-1, total PYY, leptin and insulin) were measured before and after each study period.

Results: There was no differences between the prebiotic- and placebo period in subjective appetite sensations (hunger, satiety, fullness and PFC) or food intake. PYY had a significantly greater change from baseline at 60 min postprandially after treatment with placebo compared with prebiotics (16.0 pg/mL (P_{25}, P_{75}: -7.9, 42.0) vs. -3.1 pg/mL (P_{25}, P_{75}: -20.7, 21.1) [$p<0.01$] respectively). However, carryover effects were found for PYY, and the significant effect was abolished when analyzing PYY in parallel samples. There were no differences in change from baseline between the prebiotic- and placebo period in concentrations of ghrelin, GLP-1, leptin or insulin.

Conclusions: Prebiotic fiber supplement did not affect subjective or objective appetite markers in people with T2DM.

Relevance: Overweight and obesity are risk factors for developing T2DM and diabetes complications. Reduced hunger and increased satiety would potentially reduce the risk of overeating and subsequent weight gain. Strategies to reduce weight gain and possibly obtain weight loss would be beneficial to reduce the risk of developing T2DM and its macro- and microvascular complications.

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Abbreviations

AG:	Acyl ghrelin
AGRP:	Agouti-related protein
ARC:	Arcuate nucleus
AUC:	Area under the curve
BBB:	Blood-brain barrier
BMI:	Body mass index
CART:	Cocaine and amphetamine related transcript
CCK:	Cholecystokinin
CV:	Coefficient of variation
DPP-4:	Dipeptidyl peptidase-4
FOS:	Fructo-oligosaccharides
GHS-R:	Growth hormone secretagogue receptor
GI:	Gastrointestinal
GLP-1:	Glucagon-like peptide-1
GLP-2:	Glucagon-like peptide-2
GPCR:	G-protein coupled receptor
LPS:	Lipopolysaccharide
MTT:	Meal tolerance test
NPY:	Neuropeptide Y
NTS:	Solitary tract
OUH:	Oslo University Hospital
PFC:	Prospective food consumption
POMC:	Pro-opiomelanocortin
PYY:	Peptide YY
SCFA:	Short chain fatty acid
SGLT-2:	Sodium-glucose co-transporter 2
T2DM:	Type 2 diabetes mellitus
VAS:	Visual analogue scale
W:	Weeks

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1 Background

1.1 Introduction

According to the International Diabetes Federation, approximately 415 million people, or 8.8% of the world's population, were living with diabetes in 2015 (1). Around 90% of these individuals have type 2 diabetes mellitus (T2DM) (1). These numbers are expected to keep rising. If the International Diabetes Federations predictions are correct, approximately 642 million people will be living with diabetes by 2040 (1). T2DM is strongly associated with obesity, comorbidities, e.g. kidney failure, blindness and cardiac problems, and overall mortality (1-3). Hence, T2DM is an enormous burden worldwide - for individuals, their families and the health care systems (1). However, the risk of T2DM and its cardiovascular complications can be reduced with a healthy lifestyle (4-7). Among the various lifestyle recommendations given, an increased intake of dietary fibers is suggested to be important in both prevention of T2DM (8, 9) and of diabetes complications in established diabetes (10).

Dietary fibers are carbohydrates that escape digestion in the upper gastrointestinal (GI) tract. Some work as bulking agents and has the ability to delay gastric emptying, reduce gut transit time and attenuate glucose absorption rate, whereas others are fermented by specific bacteria in the colon (11-13). Studies have shown a negative correlation between a high consumption of whole grain and dietary fibers and the development of T2DM (8, 9). There is also evidence that a high consumption of dietary fibers leads to better glycemic control in people already diagnosed with T2DM (14). Part of the protective effect of dietary fibers may be due to the delayed gastric emptying and slower uptake of glucose from the intestine, resulting in more stable blood glucose levels. However, anaerobic fermentation of fibers in the colon is also thought to play a protective role in the development and control of T2DM (14).

Bacteria in the gut, also called the gut microbiota, are responsible for the fermentation of dietary fibers in the cecum and colon (15). Fermentation leads to production of short chain fatty acids (SCFAs), such as propionate, butyrate and acetate (12). SCFAs are shown to have several beneficial effects for the host. These include regulating immune response, improving the gut epithelial barrier function and preventing bacterial translocation (16, 17). There is also evidence that SCFAs affect appetite in healthy people through interaction with G-protein coupled receptors (GPCRs). This interaction leads to regulation of the appetite related

hormones ghrelin, cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (11, 18-23). Hence, SCFAs contribute to regulation of appetite in healthy subjects.

People with T2DM are shown to have a moderate degree of imbalance of the gut microbiota (24). Studies have shown that an increased intake of prebiotic fibers can improve imbalance of gut bacteria in healthy individuals. Intake of prebiotic fibers led to increased feeling of satiety and reduced feeling of hunger, decreased food intake, increased levels of GLP-1, GLP-2 and PYY and decreased levels of ghrelin in these studies (25-28). However, no studies have investigated the effect of prebiotic fiber supplements on appetite and appetite regulating hormones in people with T2DM. Thus, the aim of this study was to investigate how intake of prebiotics affects subjective and objective appetite markers and food intake in people with T2DM.

1.2 Theoretical background

1.2.1 Appetite and appetite regulation

Appetite and food intake are controlled by a complex interaction between environmental and physiological factors. Environmental factors, such as availability, time of day and cost etc., affect food intake and create meal-to-meal variations in energy intake (29). Physiological signals can be both tonic (long-term) and episodic (short-term), and regulate appetite, food intake and metabolism both centrally and peripherally in a homeostatic manner. Tonic signals reflect the chronic nutritional status and include hormones, such as leptin and insulin, secreted from adipose tissue and the pancreas respectively. Episodic signals reflect the acute nutritional status, and include the hormones ghrelin, PYY, GLP-1 and cholecystokinin (CCK) secreted from the GI tract (30). These appetite regulating hormones can work either directly through stimulation of the hypothalamus, or indirectly via stimulation of vagal afferent nerves that project the solitary tract (NTS) and thereby stimulate the hypothalamus **(Figure 1)** (30). Reward-based, hedonic (non-homeostatic) aspects of food intake also interact with the homeostatic regulation (30-33).

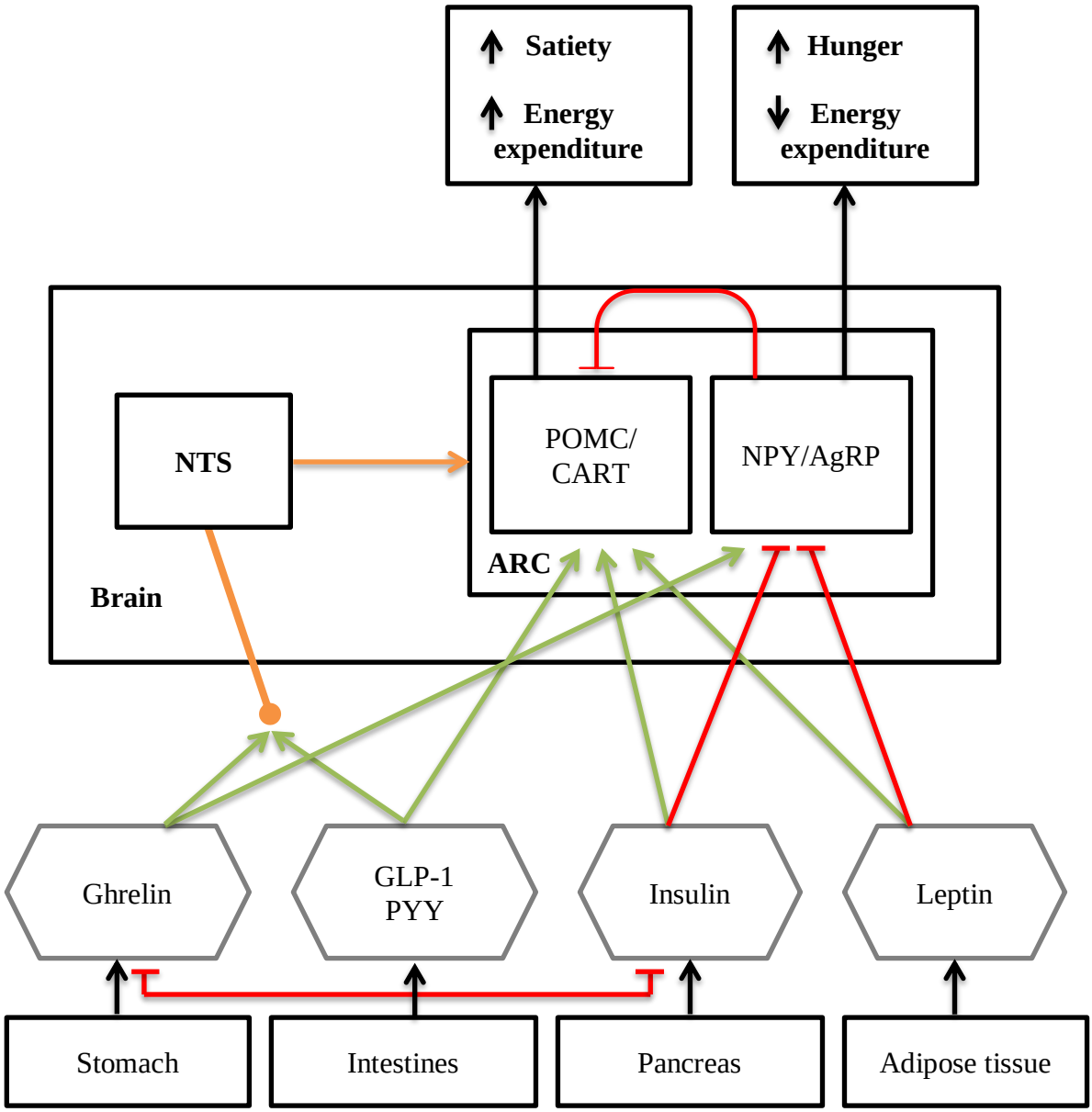

Figure 1. Overview of appetite regulation.
Green arrows: stimulation; red lines: inhibition; yellow line: vagal afferent nerves; yellow arrow: neurons projecting the hypothalamus.
AgRP: agouti-related protein; ARC: arcuate nucleus; CART: cocaine and amphetamine related transcript; GLP-1: glucagon-like peptide-1; NPY: neuropeptide Y; NTS: solitary tract; POMC: pro-opiomelanocortin; PYY: peptide YY.

Hypothalamic regulation of appetite

The hypothalamus is a region of the brain, important in the regulation of food intake and energy expenditure. In particular, the arcuate nucleus (ARC) within the hypothalamus is a key regulator of appetite and metabolism (34). There are two types of neuronal populations

within the ARC: orexigenic neurons, which increases food intake and reduces energy expenditure, and anorexigenic neurons, which decreases food intake and increases energy expenditure. The orexigenic neurons consists of neuropeptide Y (NPY) and agouti-related protein (AgRP)-expressing NPY/AgRP neurons, whereas the anorexigenic neurons consist of pro-opiomelanocortin (POMC)-expressing and cocaine and amphetamine related transcript (CART)-expressing POMC/CART neurons (35-37). These regions of the hypothalamus are close to fenestrated capillaries, which make the blood-brain barrier (BBB) "leaky". The leaky BBB makes it possible for peripheral hormones and nutrient signals to reach the ARC. Peripheral and central neuronal signals, such as signals from vagal afferent nerves and the NTS respectively, also give inputs to the hypothalamus. The hypothalamus then generates a coordinated feedback response to the peripheral and central signals, regulating appetite and energy expenditure (33).

Appetite hormones

The GI tract is the largest endocrine organ in the human body (38). It has specialized enteroendocrine cells that release appetite regulating hormones in response to GI distention and nutritional content. In a fasted state the GI tract releases the orexigenic hormone ghrelin, while in a fed state it releases the anorexigenic hormones GLP-1, GLP-2, CCK and PYY. The pancreas and the adipose tissue also release satiety stimulating hormones, such as insulin and leptin respectively (30) **(Figure 1)**.

Ghrelin is an orexigenic peptide hormone stimulating appetite and food intake (39). It is secreted by closed-type enteroendocrine cells, mainly in the gastric fundus (39, 40). Relatively little is known about the exact molecular pathways regulating ghrelin secretion, though it is suggested that both neural, hormonal and nutrient signals, as well as food cues modulate secretion (41). Blood concentration of ghrelin is high before meals, decreases fast after meals, and increases gradually until next meal (42-44). There are two types of ghrelin: acyl ghrelin (AG) and des-acyl ghrelin (45). O-acyltransferase catalyzes the conversion of des-acyl ghrelin to AG, a conversion necessary for AG's binding to the growth hormone secretagogue receptor (GHS-R) and its orexigenic effects (39, 44, 46). Ghrelin modulates appetite in two different ways: via circulation, crossing of the BBB and stimulation of GHS-R in the ARC and via the stimulation of vagal afferent nerves projecting the NTS (46-49) **(Figure 1)**. In addition to appetite stimulation, ghrelin also stimulates gastric acid secretion and gastric motility, and inhibits insulin secretion from pancreatic β-cells (47, 50).

GLP-1 is an anorexigenic, incretin hormone secreted by enteroendocrine cells mainly in the jejunum, ileum and colon (39, 40). Secretion of GLP-1 is stimulated when macronutrients reach the intestines and stimulates receptors on the apical surface of specific enteroendocrine cells (39, 40). GLP-1 levels are low in fasting and increase after meals, stimulating satiety (39, 51-54). GLP-1 is rapidly deactivated by dipeptidyl peptidase-4 (DPP-4) after entering the blood stream, hence, only a small fraction of plasma GLP-1 is active (39, 51, 54). GLP-1 modulates appetite through stimulation of vagal afferent nerves reaching the NTS (54). A second way GLP-1 may modulate appetite is via circulation, crossing of the BBB and stimulation of the ARC (38, 39) **(Figure1)**. In addition to stimulating sateity, GLP-1 is important for energy homeostasis and glycemic control by increasing insulin secretion, decreasing glucagon secretion and inhibiting gastric emptying (38, 39).

Peptide YY (PYY) is an anorexigenic hormone secreted by enteroendocrine cells in the distal small intestine and colon, often co-expressed with GLP-1 (38, 39, 55). It is present in two forms: PYY(1-36) and PYY(3-36). PYY(1-36) is the secreted form, whereas PYY(3-36) is the active form resulting from cleavage of PYY(1-36) by DPP-4 (39, 55). PYY is low in fasting, increases after meals and stays elevated for hours before slowly decreasing until the next meal (39, 43, 55). Secretion is stimulated by nutrients reaching nutrient receptors on the apical surface of the enteroendocrine cells (38, 39). PYY(3-36) is suggested to modulate appetite in two ways: 1) by reaching circulation, crossing the BBB and stimulating Y2-receptors in the ARC, or 2) by stimulating Y2-receptors on vagal afferents and thereby affecting the NTS (54-56) **(Figure1)**. In addition to stimulating satiety and reducing food intake, PYY(3-36) also contributes to energy homeostasis and reduces gastric acid secretion, gastric emptying and GI motility (38, 56).

Insulin is a pancreas-derived peptide hormone secreted from β-cells of the islets of Langerhans in response to nutrient ingestion (33). Plasma insulin is positively correlated with body weight and amount of adipose tissue, in addition to recent food intake (34, 36, 37). Insulin is important in glucose homeostasis peripherally, and in the regulation of food intake and energy homeostasis at the level of the hypothalamus (33). It modulates appetite by crossing the BBB and stimulating anorexigenic POMC neurons and suppressing orexigenic NPY/AgRP neurons in the ARC (33, 36) **(Figure1)**. Central and peripheral processes lead to

increased metabolic activity and glucose homeostasis by e.g. suppressing hepatic glucose production and increasing glucose uptake in muscle and adipose tissue (33, 36, 57). Insulin also inhibits ghrelin secretion (50).

Leptin is secreted from adipose tissue in proportion to body fat content (33, 34, 37). It modulates appetite and energy expenditure by crossing the BBB and suppressing orexigenic NPY/AgRP neurons and stimulating anorexigenic POMC neurons in the ARC (33, 37) **(Figure1)**. Leptin also influence other brain regions, such as the dorsomedial hypothalamus and the ventromedial hypothalamus, which synergistically leads to its effects on metabolism (58). Leptin has an important role in inhibiting food intake and increasing energy expenditure (33, 58). It increases body temperature, heart rate and blood pressure and it plays a role in glycemic control by suppressing hepatic glycogenolysis (34, 58).

GLP-2 and *CCK* are anorexigenic hormones released from the gut in response to food intake (30). GLP-2 contributes to cell growth and gut barrier function, whereas CCK stimulates satiety, pancreatic secretion and gallbladder contraction, inhibits gastric emptying, and modifies intestinal motility (59, 60). GLP-2 and CCK were not analyzed in this thesis and will therefore not be described any further.

1.2.2 Microbiota

The human GI tract is colonized by trillions of microorganisms called the gut microbiota (61, 62). The gut microbiota consist of bacteria, viruses and fungi (62). Although there is some discussion about the exact numbers, metagenomic sequencing has shown that there exists between 1000 and 1150 prevalent gut bacterial species, and that each individual harbors at least 160 of these species (24, 63). Different bacteria species have different features. Gut microbiota has a great variety of functional properties in human physiology, including influencing host immune system, energy generation, the production of vitamin K and some B-vitamins, xenobiotic and drug metabolism, and intestinal barrier function (61, 63, 64).

Environmental factors contribute in shaping the gut microbiota. Mode of delivery (vaginal or cesarean), type of infant feed (breast milk or formula), diet and the use of antibiotics are all factors influencing the composition of each individual's microbiota (64, 65). A diet rich in

fruit, vegetables and fibers are associated with a more diverse and richer microbiota than e.g. a Western diet high in animal products, sugars and starch (64).

Fermentation

Human intestines are unable to digest most complex carbohydrates and plant polysaccharides, due to the lack of enzymes that can hydrolyze β(2→1) glycosidic linkages (11, 15, 66). When carbohydrates and other compounds enter the colon undigested, colonic bacteria break down these compounds, allowing the process called fermentation (67). Fermentation is the process where organic compounds, including carbohydrates, are metabolized to end products, e.g. SCFAs and gases (12). Fermentation is possible because gut bacteria possess an extensive set of enzymes, including glycoside hydrolases, glycosyl transferases, polysaccharide lyases, and carbohydrate esterases, able to hydrolyze β(2→1) glycosidic linkages in dietary fibers (15, 64). Fermentation of carbohydrates depends on the physical and chemical properties of the carbohydrates, fiber dosage and each individuals composition of the gut microbiota (15).

Fermentative end products

The main end products of bacterial fermentation of dietary fibers are SCFAs, mainly butyrate, propionate and acetate, lactate and gases (H_2, CO_2 and CH_4) (15, 16, 64, 67-69). The end products have various beneficial effects to the host. SCFAs directly activate GPCRs, inhibit histone deacetylases and serve as an energy substrate (66, 67). Ninety-five percent of the SCFAs are absorbed in the colon. Production of SCFAs therefore leads to increased energy absorption and contribute with approximately 200 kcal/day (70). The activation of GPCRs causes a number of downstream events depending on the cell type, whereas inhibition of histone deacetylases influences gene expression (66). Through these pathways SCFAs influence immune function, gastrointestinal epithelial cell integrity, glucose homeostasis, lipid metabolism, and appetite regulation (67). Both lactic acid and SCFAs reduce colonic pH, which support the growth of butyrate-producing bacteria (15).

Different SCFAs have different physiological features. *Butyrate* is an important energy source in enterocytes and colonocytes (71). It also regulates immune response and causes macrophages to reduce their production of proinflammatory mediators, thereby preventing macrophages from destroying the intestinal microbiota (17). *Propionate* can be converted to

glucose by gluconeogenesis locally in the enterocytes and colonocytes, or in the liver (71, 72). *Acetate* is found in high concentrations in blood. It serves as a substrate for *de novo* lipogenesis in the liver (72, 73) and reduces gut permeability (16). Like butyrate, acetate also contributes to regulation of immune response, however, probably beyond the gut due to its high concentration in the peripheral circulation (16).

Type 2 diabetes and microbiota

In addition to established risk factors for T2DM, such as genetic predisposition, low physical activity and obesity, an altered microbiota has emerged as a factor that may be linked to T2DM (24). Studies on microbial genome have showed that individuals with T2DM have a moderate degree of gut microbial dysbiosis (24). Microbial dysbiosis describes a state where the balance of the normal microbiota has been disturbed. Individuals with T2DM have reduced levels of butyrate-producing bacteria and increased levels of various opportunistic pathogens (24, 74). People with T2DM are also shown to have higher blood concentrations of lipopolysaccharide (LPS). LPS is a component of the cell wall of gram-negative bacteria, which promotes secretion of pro-inflammatory cytokines and is associated with low-grade inflammation in people with T2DM (24, 60). The increased LPS concentration is related to higher prevalence of pathogenic bacteria in the gut, accompanied with increased gut permeability due to poorly functioning tight junctions (60).

1.2.3 Dietary fibers

Dietary fibers and prebiotics

Dietary fibers are carbohydrates that cannot be digested by enzymes in the human upper GI tract. Fibers are originally thought of as components that increase fecal bulk and affects transit time. However, some fibers are also utilized by specific gut bacteria through fermentation (11, 12). Dietary fiber is a broad term, and the impact on microbiota varies based on the type of fiber (15). Some fibers have prebiotic effects, meaning that they have the ability to selectively alter the composition or metabolism of the microbiota in a beneficial fashion (12, 15, 75). The selective properties of the prebiotic fibers are seen through the growth of bacteria associated with improved health, such as Bifidobacteria and Lactobacilli, at the expense of other groups of non-beneficial bacteria in the gut (11, 12, 75). Fibers have to fulfill three criteria to be classified as prebiotic: (1) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, (2) fermentation by intestinal

microbiota, and (3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing (76). Fibers that can be classified as prebiotic include inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides among others (75). Two of the most studied and well-established prebiotics are inulin and FOS (11, 68).

Inulin-type fructans

Inulin and FOS consist of linear fructose chains bound together by β(2→1) glycosidic linkages, typically with a terminal glucose unit (11, 77). Inulin usually occurs with a variety of chain lengths with up to 60 fructose units. FOS is a shorter kind of inulin-type fructan, with chain lengths of 2-10 fructose units (77). Endogenous enzymes in the human intestines are unable to hydrolyze these linkages. Hence, inulin-type fructans passes the small intestine undigested and are fermented by gut microbiota (66). Fermentation of prebiotic fibers is shown to provide health benefits, whereas a low fiber intake is purported to be a driver in microbiome depletion and to increase the risk of non-communicable diseases (78).

Common dietary sources of inulin-type fructans are wheat, onion, banana, garlic, leek and chicory (11, 79). Inulin can also be industrially produced, usually by extraction from chicory root or synthetically prepared from sucrose. FOS can thereafter be produced by hydrolysis of inulin (11, 79). FOS is highly soluble, provides a sweet taste and possesses other qualities similar to sugar, but with fewer calories (79). Inulin is less soluble than FOS due to its longer chain length. Inulin forms microcrystals when mixed with drinks like water or milk, which form a smooth creamy texture and provide a fat-like mouthfeel (79). Because of these properties and the fact that they have no "off flavors", inulin and FOS are often added to fat- and energy reduced foods to increase acceptability, stability and mouthfeel, and at the same time increase fiber content (79).

Prebiotic fibers and health effects

Supplementation of various prebiotic fibers may lead to a number of health beneficial effects. Studies in non-diabetic humans have found that prebiotic supplements gave a normalization of the gut microbiota (80) and improved risk factors for non-communicable diseases, such as reduced weight gain (28, 81, 82), reduced body fat (82), improved blood lipid profile (80, 82), improved blood glucose regulation (27, 28) and reduced inflammation (80). Human studies have also shown an effect of prebiotics on appetite, with increased satiety (26, 82),

reduced hunger (26), increased secretion of GLP-1 and PYY (25, 27, 28) and reduced secretion of ghrelin (28). Health beneficial effects are also shown in animal studies. A study by Cani *et al.* showed that prebiotic supplements normalized the gut microbiota, reduced inflammation, improved gut barrier function, increased GLP-2 secretion and reduced food intake in obese mice (83). It has also shown that a rise in SCFAs, which may be subsequent to an increased intake of prebiotic fibers, leads to increased leptin expression and secretion in rodents (84).

1.2.4 Microbiota and appetite hormones

Nutrients stimulating nutrient receptors on enteroendocrine cells lead to the first rise in appetite hormone release, whereas SCFAs produced by the gut microbiota lead to the later phase of appetite hormone secretion (39). SCFAs exhibit a number of their actions by binding and activating GPCRs. GPCRs are widely distributed in immune cells, intestinal mucosa, liver and adipose tissue (24). By binding to GPCRs on enteroendocrine cells, SCFAs contribute to appetite regulation. This binding promotes production and secretion of appetite hormones, such as GLP-1, GLP-2 and PYY (24, 60, 85). Microbiota also deconjugates primary bile acids, hindering their recirculation in the enterohepatic circulation. The primary deconjugated bile acids are further metabolized to secondary bile acids. Secondary bile acids bind to GPCRs on enteroendocrine cells and promotes secretion of GLP-1 (24). Therefore, both the interaction between prebiotic fibers and microbiota and consequently the production of SCFAs, and microbiotas conversion of primary to secondary bile acids, increases satiety, regulates glucose- and energy homeostasis and maintains gut barrier function via the rise in GLP-1, PYY and GLP-2 (38, 60).

1.3 Objective and hypothesis

The main objective of the Fiberdia study was to investigate the effect of prebiotic fibers on signaling molecules that regulate blood glucose, appetite and energy intake in individuals with T2DM. The present master study is a part of the Fiberdia study, focusing on the effect of prebiotic fibers on appetite.

We hypothesize that the participants after intake of prebiotics compared with placebo will have a:

- reduced feeling of hunger and prospective food consumption
- increased feeling of satiety and fullness
- reduced food intake
- reduced secretion of hunger stimulating hormones (AG)
- increased secretion of satiety stimulating hormones (GLP-1, PYY, insulin and leptin)

We also hypothesize that there is a correlation between subjective (visual analogue scale (VAS) and food intake) and objective (appetite related hormones) appetite markers.

2 Methods

2.1 Permissions

The study was approved by the Committees for medical and health research ethics (case number: 2014/1180) and registered at clinicaltrials.gov (NCT02569684). All participants signed an informed consent before participating in the study **(Appendix I)**. Personnel in contact with participants had to sign a form of confidentiality. The study was conducted in accordance with the Declaration of Helsinki, concerning medical research in humans.

2.2 Study design

The Fiberdia study was designed as a double-blind randomized placebo-controlled trial with crossover design. The study consisted of two 6 weeks (w) supplementation periods separated by a 4w washout period to avoid carryover effects **(Figure 2)**. A statistician not involved in the project randomized the participants to begin with either prebiotics or placebo. A nurse not involved in the study provided the intervention supplements according to the randomization list. Participants were randomized to begin with either 16 g of prebiotics (Synergy 1, 50/50 mix of FOS and inulin) or 16 g placebo (maltodextrin) per day for 6w. After a 4w washout period, participants went through another 6w of intervention with the supplement they did not receive in the first period **(Figure 2)**. Maltodextrin was selected as placebo because of its similar taste and appearance to inulin and FOS, but with no known effects on the microbiome. The supplements were provided as powder in sachets of 8g (Beneo, Mannheim, Germany). The first week of each supplementation period participants consumed one sachet per day, whereas they consumed 2 sachets per day the following 5w. How and what time of the day the supplements were ingested was determined by each participant. Participants were told to bring back any unused sachets at the next visit as a measure of compliance. Participants were told not to purposely loose or gain weight and to maintain their normal diet and activity level throughout the study period. All participants who consumed ≥50% of the powder were eligible for inclusion in the analysis.

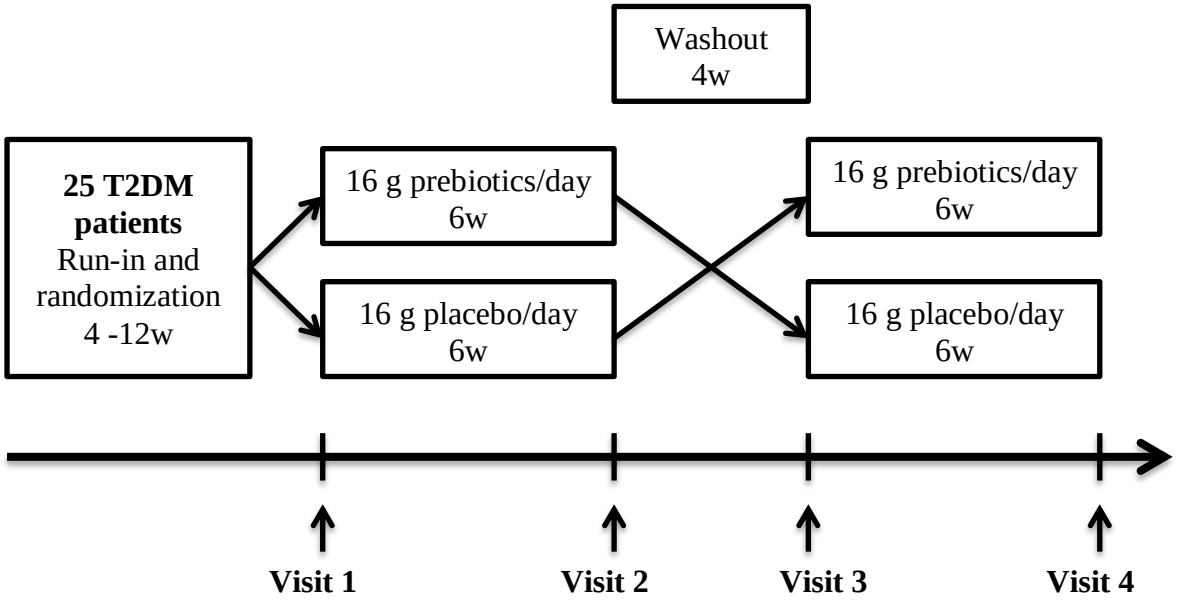

Figure 2. Flowchart of study period.
T2DM: type 2 diabetes mellitus; w: weeks.

2.3 Study population

The target population was individuals with T2DM. The participants were recruited from the diabetes outpatient clinic at Oslo University Hospital (OUH), Aker, OUH's web-page/Facebook-page, The Norwegian Diabetes Association's webpage, Facebook-page, and paper, GP/medical centers and pharmacies and a selection of local newspapers. Participants were adults (>18 years old) with a body mass index (BMI) ≤40 kg/m^2 and an HbA1c of 6.5-10.0 %. Exclusion criteria were treatment with insulin or GLP-1 analogues, pregnancy, estimated fiber intake of >25 g/day, body weight changes of >3 kg the last 3 months before baseline, high intensity exercise >3.5 hours per week, use of antibiotics, use of prebiotic- or probiotic supplements, diagnosis of cancer, chronic bowel disease, irritable bowel syndrome or dementia, high alcohol consumption (>3-4 units/day or >21 units/w) or use of narcotics.

2.4 Measurements

Data was collected at baseline (visit 1), after 6w of intervention period one (visit 2), after 4w of washout (visit 3), and after 6w of intervention period two (visit 4) **(Figure 2)**. Levels of appetite regulating hormones and amount of food eaten were measured and subjective feeling of appetite was recorded at every visit, whereas data considering palatability of test meal was only collected at visit 1. Anthropometric measures were performed at every visit. Testing

took place at OUH, Aker. Participants were told not to perform strenuous physical activity the day before testing. Participants were fasting at arrival.

2.4.1 Appetite hormones

A meal tolerance test (MTT) was performed measuring i.a. appetite related hormones and insulin levels. A study nurse performed a venipuncture placing a catheter in a superficial vein on the participant's non-dominant arm. Blood was collected in fasting and after intake of two nutritional drinks (200 ml Fresubin 2 kcal Drink vanilla and 100 ml Fresubin Jucy Drink apple, nutritional content given in **Appendix II**). Nutritional drinks were consumed within 12 minutes. Blood samples for appetite hormone analysis was collected in EDTA tubes in fasting (0 min), and then 30, 60, 120 and 180 min postprandially. To avoid degradation of GLP-1 and AG 40 µl DPP-IV inhibitors (Merck Millipore, Germany) and 40 µl protease inhibitor (Pefabloc® SC, Merck Millipore, Germany) were added to 4 ml EDTA tubes before blood collection. Blood samples were centrifuged at 3500 rpm at 4°C for 10 minutes. Plasma was then stored at -80°C until analysis. Active GLP-1, AG, total PYY and leptin content were analyzed in duplicates using Human Metabolic Hormone Magnetic Bead Panel (Metabolism Multiplex Assay, Merck Millipore, Germany) and Luminex 200 Technology (Invitrogen, Thermo Fisher, USA). The minimum detectable concentrations of the assay correspond to the following: GLP-1: 2.7 pg/mL; PYY: 41.2 pg/mL; AG: 13.7 pg/mL; and leptin: 137.2 pg/mL. The manufacturer states that the multiplex assay has an intra-assay coefficient of variation (CV) <10 %, and inter-assay CV <15 % for all the appetite related hormones. Blood samples for measuring insulin levels were collected in EDTA tubes in fasting (-15 and 0 min), and then 15, 30, 45, 60, 90, 120, 150 and 180 min postprandially. Thirty min after collection, blood samples were centrifuged at 3500 rpm for 12 min. Insulin was measured in serum using Modular Analytics E170 (Roche, Switzerland). The method used for insulin analysis is routinely used for insulin measurements at OUH. The Hormone Laboratory at OUH states that the method has an inter-assay CV ≤ 4% for insulin analysis.

2.4.2 Food intake

Approximately 3-3.5 hours after intake of the nutritional drinks the participants helped themselves to an *ad libitum* meal. The meal contained pasta mixed with meatballs and vegetables from Fjordland (Fjordland AS, Oslo, Norway). The participants were told to eat

until they did not want to eat anymore. The meal had to be consumed within 30 minutes. All the food was weighed and registered. Any leftovers on the plate was weighed and subtracted.

During the study period, the original test meal went out of production. The test meal therefore had to be replaced with a similar dish. Both meals contained pasta and meatballs and had similar nutritional content **(Appendix III)**.

2.4.3 Subjective feeling of appetite

Subjective feeling of appetite was measured using VAS forms. The English version of the VAS is a validated method that can be used to assess motivation to eat in humans (86-88). A person not involved in the study translated the VAS forms to Norwegian. Another person translated them back to English to validate the translation. There were four questions in each form. The forms were made as booklets, showing one question at each page. Participants were told to make a vertical mark on a 100 mm long line according to how strongly they felt what was asked. In each form the participants answered questions measuring hunger, satiety, fullness and prospective food consumption (PFC). The ends of the line expressed the most negative (left) and positive (right) rating **(Appendix IV).**

Measurements were performed before the *ad libitum* meal (time 0), and then 30, 60, 90 and 180 min after meal initiation. The first four forms were filled out in the test room, whereas the last (180 min) form was filled out at home/where the participant was at the time (**Figure 3)**. The participants got a reminder to fill out the last form by SMS. The distance from the left end of the scale to the vertical mark was measured in mm.

2.4.4 Palatability of the test meal

Palatability of the test meal was measured at visit 1 using a VAS form (as described above). The VAS forms were made as booklets, showing one statement at each page. There were four statements in each form measuring visual appearance, smell, taste and aftertaste of the dish. The ends of the lines represented the most positive (left) and negative (right) ratings **(Appendix V).** The distance from the left end of the scale to the vertical mark was measured in mm. Four participants had to fill this form out twice, as they got two different test meals.

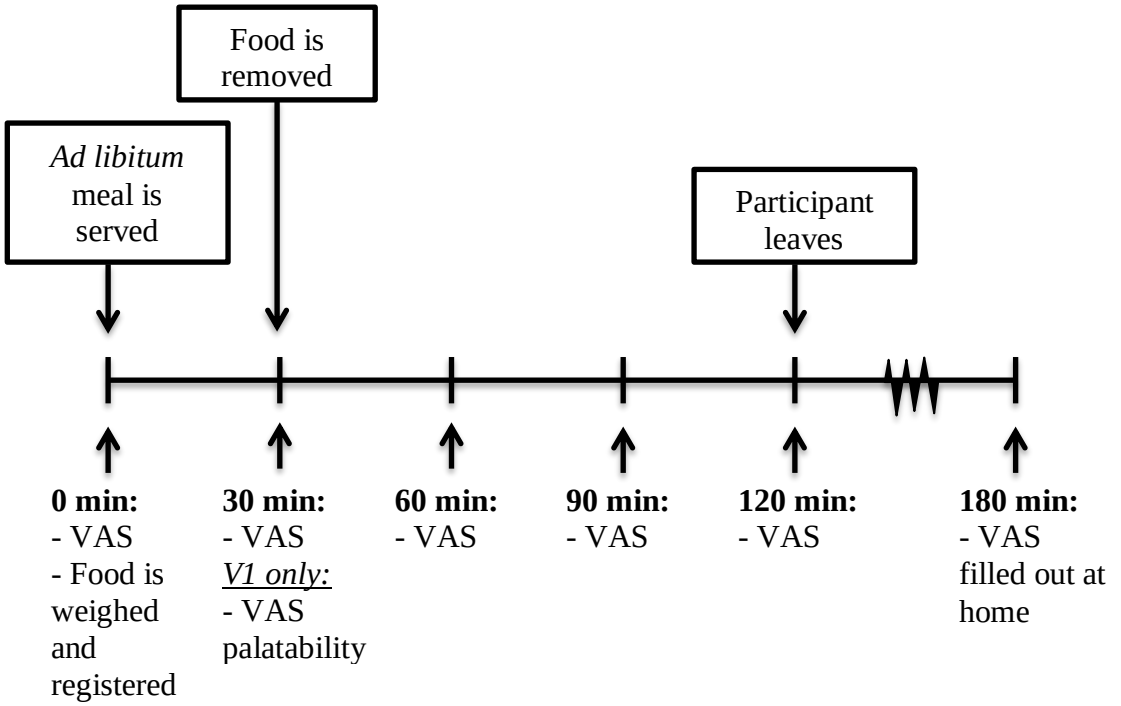

Figure 3. Timeline of appetite test.
VAS: visual analogue scale; V1: visit 1.

2.4.5 Anthropometry

Height was measured using a standard altimeter at the day of screening. Participants were not wearing shoes or any headgear while being measured. Weight, BMI and percentage of adipose tissue were measured at the beginning of every visit using a body composition analyzer (Tanita BC-418 MA Segmental Body Composition Analyzer). Participants were measured bare feet with only light clothes on. Their age and height, as well as subtraction of 1 kg clothes, were punched in to the body composition analyzer. BMI was automatically calculated.

2.5 Power calculation

Sample size in the Fiberdia study was calculated based on expected effects on the primary outcome measurement, the change in GLP-1-response to a standardized meal. Available data on the subject is scarce, but based on a study with design similar to Fiberdia (89), a statistician estimated that we needed 23 subjects to achieve 80% power at alpha=0.05. In order to compensate for some loss to follow-up and for a possible smaller treatment effect or more variability in the treatment effect than shown in the study used in our calculations, we aimed to recruit 35 participants.

2.6 Statistical analyses

Statistical analyzes were carried out using IBM SPSS Statistics 25 (SPSS In., Chicago, IL). Tables and figures were made in Microsoft® Excel for Mac, version 16.12 (Microsoft Corp., Redmond, USA). Statistical significance was assumed at $P \leq 0.05$. Assessment of histograms, QQ-plots and Shapiro-Wilk test were used to check for normality. Paired-samples t-test was used to examine differences within and between the study periods for normally distributed data, whereas the non-parametric Wilcoxon Signed Rank test was used for skewed distributed data. Within treatment effects were examined by comparing every time point before and after each study period. Between treatment effects were examined by comparing the change from baseline after both study periods with each other. Data is presented as median and percentiles (P_{25}, P_{75}), mean ± SD or mean ± SE depending on the tests used. Correlations are calculated using Spearman's Rank Order Correlation.

Total area under the curve (AUC) was calculated for subjective feeling of appetite and appetite related hormones from 0 to 180 min using the trapezoid rule ($A = \frac{y1+y2}{2}(x2 - x1)$). Handling of missing data was done differently for appetite related hormones and subjective feeling of appetite due to very different variability of the data. Missing data on VAS scores from ≤2 time points were included in the AUC analysis by calculating the average from the entire group at the given time point. Missing data for appetite related hormones at ≤2 time points were included in the AUC analysis by extrapolating the value based on the time point before and after the missing value for the given participant. In case of missing data for >2 time points, the AUC for the given visit was set as missing.

Average insulin concentration of the two fasting blood samples (collected at -15 and 0 min before MTT) was calculated and used as the fasting (0 min) value. When one of the fasting blood samples was hemolyzed, insulin concentration from the sample without hemolysis was used as the fasting value (0 min). Values below the detection limit for AG, GLP-1 and PYY were imputed as the lowest detectable value.

3 Results

3.1 Study population

Thirty-six participants were recruited and randomized to start with either prebiotics or placebo. Eleven participants did not complete the intervention: three due to use of antibiotics, two due to serious illness, one started insulin treatment, one due to use of probiotics, one declined to participate and three were discontinued for other reasons **(Appendix VI)**. None of the participants quit due to adverse events. Twenty-five participants completed the study and were included in the analyses. Baseline characteristics are given in table 1.

Table 1. Baseline characteristics of the 25 participants.

	Mean (SD)
Age (years)	63.1 (11.5)
Body weight (kg)	85.3 (18.5)
- Men	91.2 (16.5)
- Women	76.5 (18.6)
BMI (kg/m^2)	29.1 (4.7)
Time since diagnosis (years)	4.7 (4.4)
	n (%)
Gender	
- Women	10 (40)
Diabetes medication	18 (72)
- Metformin	15 (60)
- SGLT2 inhibitors	2 (8)
- DPP-4 inhibitors	5 (20)
- Sulphonylureas	1 (4)

BMI: body mass index; DPP-4: dipeptidyl peptidase-4; SD: standard deviation; SGLT2: sodium-glucose co-transporter 2.

Thirteen participants started with prebiotics, and twelve participants started with placebo.

3.2 Compliance

Participants brought unused sachets of powder back at visit 2 and 4. The sachets were counted and registered. There was no difference between the prebiotic- and the placebo period in how many sachets that were brought back (p=0.34). Mean number of unused sachets was 2.5 (95%CI: 0.8, 4.3) and 3.3 (95%CI: 1.5, 5.0) in the prebiotic- and placebo period respectively out of 77 sachets given in each period.

There was no significant weight change in either of the study periods. Mean weight change was -0.14 ± 1.0 kg in the prebiotic period, and -0.26 ± 1.1 kg in the placebo period.

3.4 Subjective feeling of appetite

Within group change

Median fasting (time 0) scores for feeling of hunger increased significantly at 6w compared with baseline in the prebiotic period (p=0.02) **(Figure 4A and Table 2).** Hunger scores were also significantly increased at 60 and 90 min postprandially after 6w of treatment with placebo compared with baseline (p=0.03 for both time points) **(Figure 4A and Table 2)**. There was no significant change in feeling of satiety or fullness from baseline to after prebiotic- or placebo period at any time point **(Figure 4B and 4C).** Median PFC scores were significantly increased 30 min postprandially at 6w compared with baseline in the prebiotic period (p=0.03) **(Figure 4D and Table 2)**.

There was no significant change in AUC from baseline to after prebiotic- or placebo period for hunger, satiety, fullness or PFC **(Figure 5).**

Difference in change between study periods

There was no statistically significant difference in change for hunger, satiety, fullness or PFC between the prebiotic- and placebo period **(Table 2).** There was no statistically significant difference in change of AUC for hunger, satiety, fullness or PFC between the study periods **(Figure 5)**.

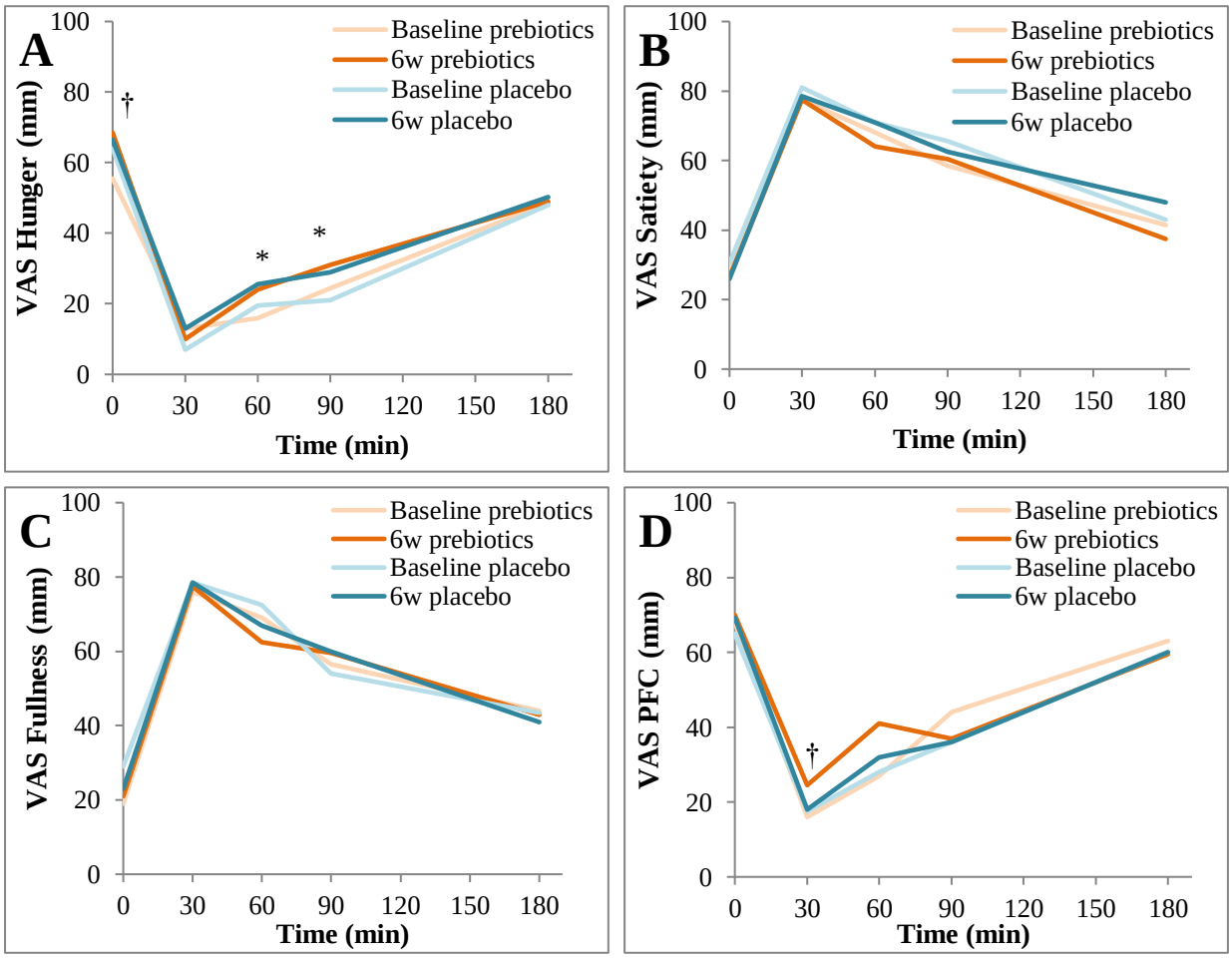

Figure 4. Feeling of **(A)** hunger, **(B)** satiety, **(C)** fullness and **(D)** PFC in fasting and at different postprandial time points.
Lines represent visits before and after a 6w prebiotic- and placebo treatment.
Data is presented as median appetite score.
PFC: prospective food consumption; VAS: visual analogue scale; w: weeks.
Symbols denote significant change from baseline (change in prebiotic period † $P \leq 0.05$; change in placebo period * $P \leq 0.05$).

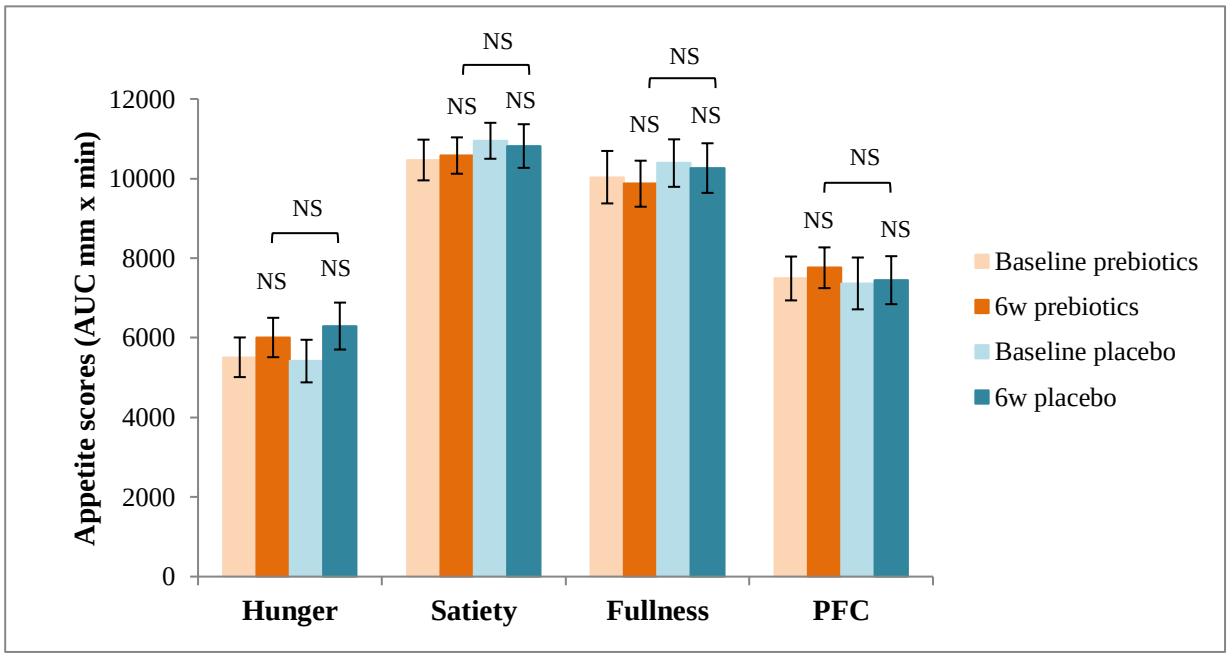

Figure 5. Area under the curve for appetite sensations.
Data presented as mean ± SE.
AUC: area under the curve; PFC: prospective food consumption; w: weeks.
Symbols above error bars denote change from baseline (NS: not significant). Symbols above brackets denote difference in change (NS: not significant).

Table 2. Change in subjective feelings of appetite from baseline to after intervention.

	Prebiotics	Placebo	
	Median (P_{25}, P_{75})	**Median (P_{25}, P_{75})**	**p[#]**
Δ Hunger (mm)			
0 min	10.5 (-1.3, 26.5)*	4.5 (-4.0, 18.3)	0.43
30 min	0.5 (-4.0, 5.0)	5 (-2.3, 11.5)	0.18
60 min	1.0 (-4.5, 9.8)	3.0 (-2.0, 13.8)*	0.15
90 min	2.5 (-2.5, 9.3)	5.0 (0.0, 15.8)*	0.52
180 min	1.5 (-5.8, 13.5)	-3.0 (-9.9, 28.1)	0.28
Δ Satiety (mm)			
0 min	1.0 (-13.8, 7.8)	3.0 (-12.3, 10.3)	0.71
30 min	-1.0 (-10.5, 7.5)	0.0 (-6.8, 6.3)	0.87
60 min	-6.0 (-13.5, 4.0)	-2.0 (-6.3, 2.5)	0.97
90 min	1.0 (-8.0, 11.8)	-3.0 (-13.0, 10.8)	0.52
180 min	1.5 (-10.5, 12.3)	8.3 (-9.6, 11.8)	0.61
Δ Fullness (mm)			
0 min	-0.5 (-8.3, 10.5)	-1.3 (-16.8, 10.1)	0.43
30 min	0.5 (-10.0, 4.3)	0.0 (-6.3, 7.8)	0.97
60 min	-2.0 (-9.0, 6.0)	-3.5 (-13.0, 4.5)	0.33
90 min	0.0 (-4.5, 12.0)	1.5 (-13.3, 9.5)	0.97
180 min	-4.5 (-12.5, 10.4)	2.5 (-20.6, 14.1)	0.87
Δ PFC (mm)			
0 min	1.5 (-7.8, 8.5)	3.8 (-3.5, 8.0)	0.68
30 min	5.0 (-1.5, 11.5)*	-1.0 (-12.5, 7.0)	0.07
60 min	4.0 (-4.8, 19.0)	3.0 (-3.5, 12.8)	0.48
90 min	-2.0 (-19, 8.3)	1.5 (-14.3, 15.8)	0.70
180 min	3.0 (-18.8, 15.6)	-3.5 (-9.8, 7.3)	0.86

Data presented as median and percentiles (P_{25}, P_{75}).
PFC: prospective food consumptions; w: weeks.
denote between group differences, * denote $p \leq 0.05$ within group change from baseline.

3.5 Food intake

Intake of *ad libitum* meal was weighed and registered at every visit. There was no significant change in food intake from baseline to 6w either of the study periods **(Figure 6)**. There was no significant difference in change from baseline to 6w between the prebiotic- and the placebo period (p=0.86) **(Figure 6)**.

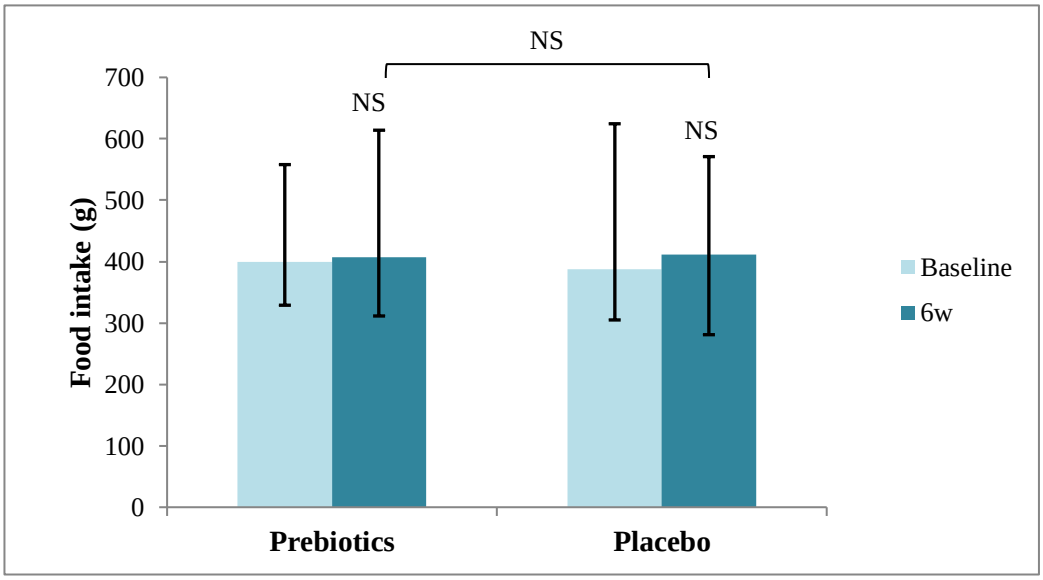

Figure 6. Food intake before and after the study periods.
Data is presented as median and percentiles (P_{25} and P_{75}).
W: weeks.
Symbols above error bars denote change from baseline (NS: not significant).
Symbols above brackets denote difference in change (NS: not significant).

3.6 Appetite hormones

Assay variation

Intra-assay CV was ≤ 20% for AG, GLP-1 and PYY and ≤ 15% for leptin, and inter-assay CV was ≤ 10% for all appetite related hormones in our study measured with the multiplex assay.

Within group change

Median plasma concentration of AG was significantly lower 60 min postprandially after 6w of treatment with placebo compared with baseline (p=0.02) **(Figure 7A and Table 3)**. There was no significant change in GLP-1 or leptin from baseline to 6w at any time point in either of the study periods **(Figure 7B and 7C).** Median plasma concentration of PYY was significantly increased at 60, 120 and 180 min postprandially at 6w compared with baseline after treatment with placebo (p=0.01, p=0.04 and p=0.02 respectively) **(Figure 7D and Table 3)**. Median plasma concentration of insulin was significantly decreased at 150 min postprandially after 6 w of treatment with prebiotics compared with baseline (342.0 pmol/L (P_{25}, P_{75}: 250.0, 485.5) vs. 398.0 pmol/L (P_{25}, P_{75}: 320.0, 490.5) respectively [p=0.03]) **(Figure 7E)**.

AUC for PYY was significantly increased after 6w with placebo treatment compared with baseline **(Figure 8D)**. There was no significant effect of either prebiotic- or placebo treatment on plasma AG-, GLP-1-, leptin-, or insulin concentrations when inspecting AUC **(Figure 8A-C and 8E)**.

Difference in change between study periods

There was no statistically significant difference in change from baseline for AG, GLP-1, leptin or insulin between the study periods **(Table 3)**. There was a significantly greater change from baseline to 6w in PYY levels at 60 min postprandially in the placebo- compared with the prebiotic period **(Table 3).**

There was no difference in change between the study periods for any of the appetite related hormones when inspecting AUC **(Figure 8A-E)**.

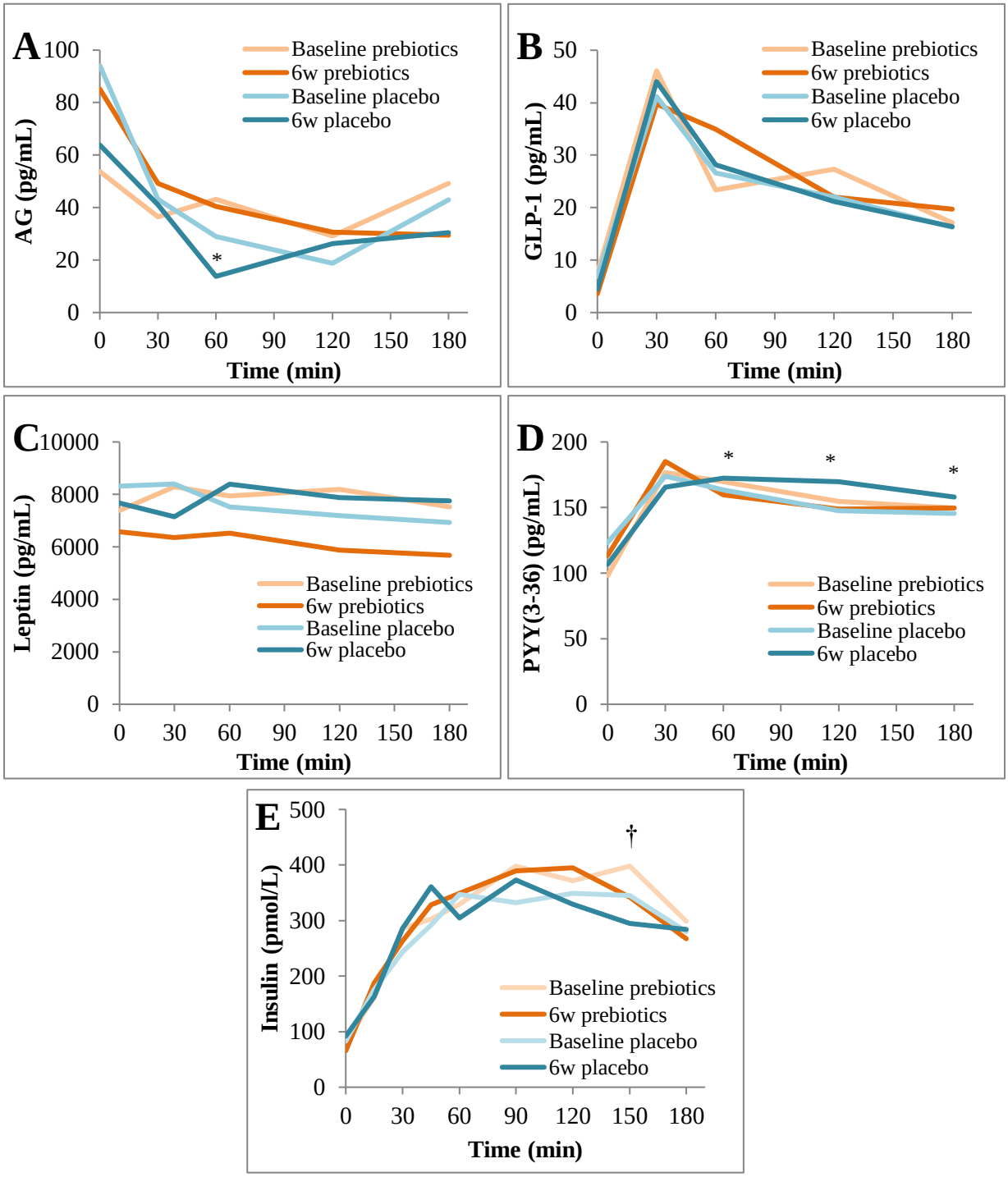

Figure 7. Plasma concentration of **(A)** AG, **(B)** GLP-1, **(C)** PYY, **(D)** leptin and **(E)** insulin at different postprandial time points before and after both study periods.
Data is presented as median hormone concentration.
AG: acyl ghrelin; GLP-1: glucagon-like peptide 1; PYY: peptide YY; w: weeks.
Symbols denote significant changes from baseline (change in prebiotic period † P≤0.05, change in placebo period * P≤0.05).

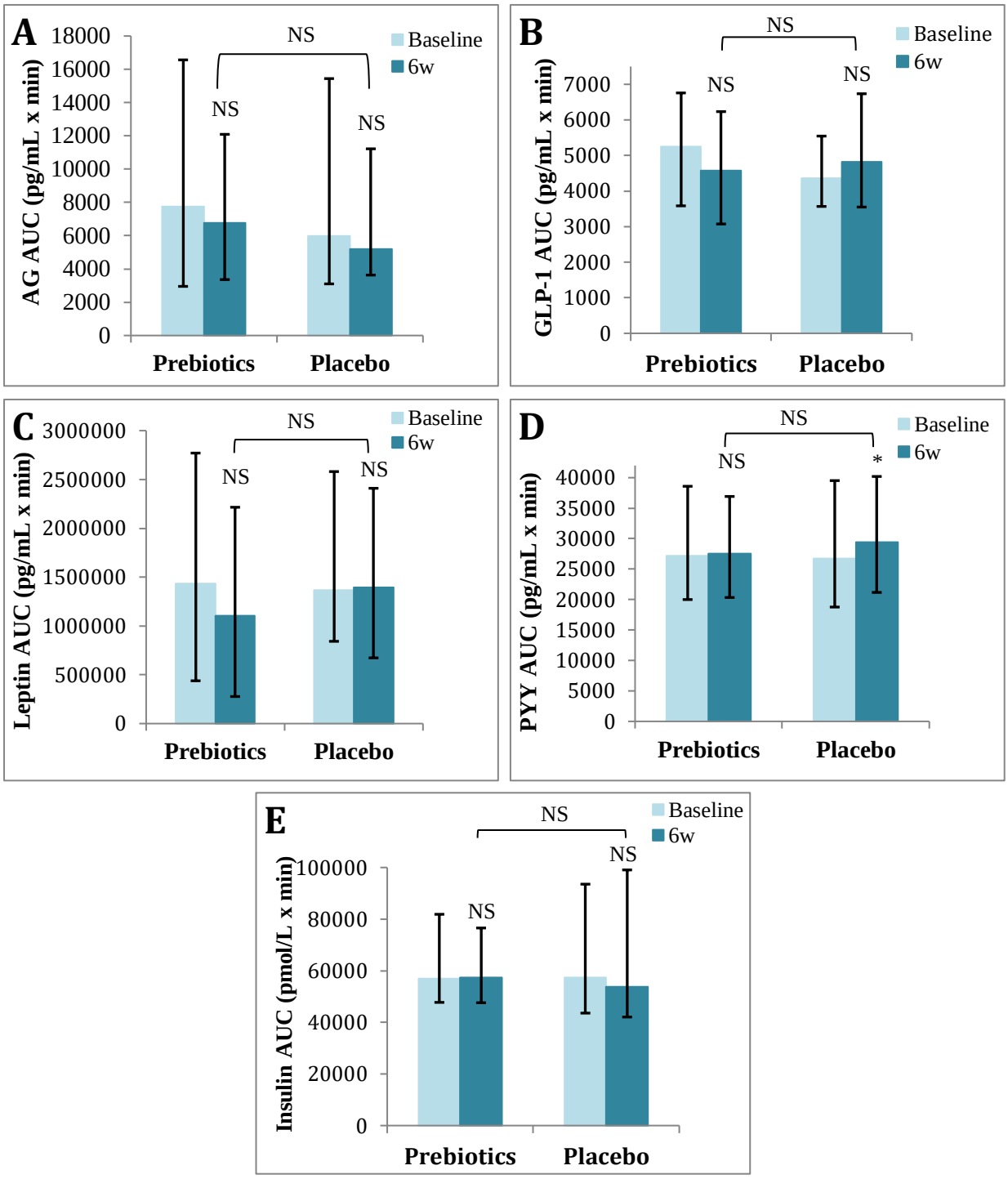

Figure 8. AUC for **(A)** AG, **(B)** GLP-1, **(C)** PYY, **(D)** leptin and **(E)** insulin before and after study periods.
Data presented as median and percentiles (P_{25} and P_{75}).
AG: acyl ghrelin; AUC: area under the curve; GLP-1: glucagon-like peptide 1; PYY: peptide YY; w: weeks.
Symbols above error bars denote significant change from baseline (* $P \leq 0.05$; NS: not significant). Symbols above brackets denote difference in change (NS: not significant).

Table 3. Fasting and postprandial baseline plasma concentrations and change from baseline after 6w in appetite related hormones.

	Prebiotics		Placebo		
	Baseline	Δ6w	Baseline	Δ6w	
	Median (P_{25}, P_{75})	Median (P_{25}, P_{75})	Median (P_{25}, P_{75})	Median (P_{25}, P_{75})	p[#]
AG (pg/mL)					
0 min	53.7 (17.8, 157.6)	-4.4 (-36.5, 45.2)	94.1 (34.8, 152.0)	-2.6 (-60.6, 32.8)	0.48
30 min	36.5 (13.7, 97.4)	-3.7 (-17.8, 18.2)	43.2 (22.3, 99.1)	-6.3 (-32.1, 21.8)	0.56
60 min	43.2 (13.7, 79.2)	-2.8 (-24.1, 8.7)	28.9 (13.7, 70.3)	-5.0 (-14.0, 1.1)*	0.88
120 min	29.2 (13.7, 75.8)	0 (-10.3, 15.5)	18.8 (13.7, 65.6)	0.0 (-9.7, 11.6)	0.97
180 min	49.1 (13.7, 98.9)	0 (-13.7, 13.2)	42.9 (13.7, 94.4)	-2.0 (-31.7, 31.1)	0.91
GLP-1 (pg/mL)					
0 min	7.5 (4.1, 14.8)	-1.7 (-6.3, 2.0)	6.9 (2.7, 11.5)	-1.1 (-4.9, 1.5)	0.99
30 min	46.1 (30.6, 58.7)	-11.9 (-19.1, 2.8)	41.2 (26.2, 58.5)	5.3 (-9.2, 21.6)	0.12
60 min	23.4 (19.4, 38.9)	2.9 (-7.0, 7.5)	26.6 (19.8, 41.7)	1.5 (-8.7, 8.4)	0.66
120 min	27.3 (15.3, 32.4)	-0.8 (-16.4, 6.8)	22.0 (15.4, 26.1)	4.5 (-4.4, 10.5)	0.23
180 min	17.2 (12.3, 23.6)	-1.9 (-5.0, 4.6)	16.2 (11.2, 21.9)	3.0 (-3.8, 7.4)	0.59
Leptin (pg/mL)					
0 min	7387.5 (2452.6, 16353.0)	-100.4 (-3292.5, 2375.9)	8319.0 (3582.1, 13823.0)	-6.5 (-1272.1, 601.2)	0.31
30 min	8293.0 (2662.2, 16064.4)	-140.5 (-3495.6, 1471.5)	8396.8 (3061.1, 14492.7)	-421.9 (-2092.0, 365.5)	0.81
60 min	7948.8 (2356.6, 15165.9)	190.8 (-2274.7, 2145.0)	7514.6 (2971.8, 13640.0)	82.0 (-684.8, 1130.3)	0.62
120 min	8183.2 (2395.4, 14827.3)	96.1 (-1314.9, 2335.4)	7196.1 (2996.3, 14320.5)	-346.8 (-1925.0, 363.4)	0.08
180 min	7519.6 (2342.9, 14823.1)	168.4 (-1774.0, 2164.0)	6930.0 (2925.9, 12008.6)	33.0 (-1371.8, 800.4)	0.22
PYY (pg/mL)					
0 min	98.2 (65.2, 164.3)	0.0 (-7.2, 18.0)	123.4 (48.6, 215.3)	0.0 (-28.1, 22.3)	0.18
30 min	176.7 (119.4, 234.5)	-3.1 (-24.7, 23.2)	173.9 (126.0, 228.1)	2.8 (-22.5, 30.6)	0.63
60 min	***169.9 (122.2, 218.5)***	***-3.1 (-20.7, 21.1)***	***163.3 (107.8, 210.1)***	***16.0 (-7.9, 42.0)****	***0.008***
120 min	154.8 (112.1, 207.7)	-3.7 (-31.7, 19.5)	147.6 (102.4, 210.3)	15.6 (-11.4, 39.1)*	0.17
180 min	149.6 (98.3, 218.3)	0.3 (-19.0, 15.8)	145.3 (95.3, 210.8)	12.7 (-4.5, 28.0)*	0.19
Insulin (pmol/L)					
0 min	83.0 (64.5, 112.0)	-3.5 (-27.8, 14.0)	83 (45.8, 129.5)	7.5 (-21.3, 21.5)	0.35
30 min	286.0 (180.5, 409.5)	-9.5 (-104.0, 72.5)	244 (189.0, 337.0)	27.0 (-56.6, 217.0)	0.20
60 min	329.0 (250.0, 505.0)	46 (-86.5, 129.0)	347 (255.0, 517.0)	17.0 (-90.5, 74.5)	0.64
120 min	372.0 (313.5, 598.5)	-26 (-142.0, 37.0)	349 (286.0, 586.0)	-20 (-118.9, 106.5)	0.44
180 min	299.0 (231.0, 395.5)	-22 (-110.0, 35.5)	280 (219.5, 602.0)	-42.0 (-172.5, 60.0)	0.18

Data is presented as median and percentiles (P_{25}, P_{75}).
AG: acyl ghrelin; GLP-1: glucagon-like peptide 1; MTT: meal tolerance test; PYY: peptide YY; w: weeks.
denote between group differences, * denote p≤0.05 within group change from baseline.

3.7 Carryover effect

When comparing baseline values, carryover effects were found for PYY for subjects who started with prebiotics (n=13). Fasting values of PYY were significantly higher before their placebo period compared with their prebiotic period (130.1 pg/mL (P_{25}, P_{75}: 109.7, 258.2) vs. 114.8 pg/mL (P_{25}, P_{75}: 90.3, 210.4) respectively [$p<0.01$]). This indicates that effects of prebiotic treatment lasted longer than the 4w washout period. Statistical analyses were therefore performed in parallel samples using Mann-Whitney U test.

There were no significant changes in PYY after 6w with prebiotics compared with baseline. PYY increased significantly after treatment with placebo at 60, 120 and 180 min postprandially compared with baseline. There was no difference in change between the prebiotic and placebo period **(Table 4)**.

There was no significant change in AUC from baseline to 6w in either of the study periods, and no difference in change between the periods.

Table 4. Change in PYY concentrations when analyzed in parallel groups.

	Prebiotic (n=13)	**Placebo** (n=12)	
	Median (P_{25}, P_{75})	**Median** (P_{25}, P_{75})	**p**[#]
Δ PYY (pg/mL)			
0 min	11.8 (-5.7, 43.8)	2.1 (-4.2, 35.1)	0.81
30 min	3.5 (-24.7, 47.9)	-6.6 (-22.2, 19.6)	0.89
60 min	-3.4 (-24.4, 22.9)	17.6 (-2.4, 46.4)*	0.10
120 min	-11.2 (-39.6, 24.5)	25.0 (0.7, 42.9)*	0.09
180 min	-2.5 (-18.5, 2.9)	18.0 (1.3, 35.8)*	0.11

Data is presented as median and percentiles (P_{25}, P_{75}).
PYY: Peptide YY.
denote between group differences, * denote $p \leq 0.05$ within group change from baseline.

3.9 Correlations

Food intake was significantly correlated with pre-meal scores for subjective feeling of hunger (r_s=0.55 [p=0.004]), satiety (r_s=-0.58 [p=0.002]) and PFC (r_s=0.71 [p≤0.001]), but not fullness **(Figure 9A-D)**. There were no significant correlations between food intake and how the participants perceived the meal's visual appearance, smell, taste or aftertaste.

There was a significant inverse correlation between pre-meal scores for GLP-1 and fullness (r_s=-0.41 [p=0.04]). There were no significant correlations between AG, leptin, PYY or insulin and feeling of hunger, satiety, fullness or PFC in fasting. No correlations were seen between any of the appetite related hormones and food intake.

Body fat was significantly correlated with concentrations of leptin (r_s=0.84 [p<0.001]) and insulin (r_s=0.55 [p<0.01]) **(Figure 10A-B)**.

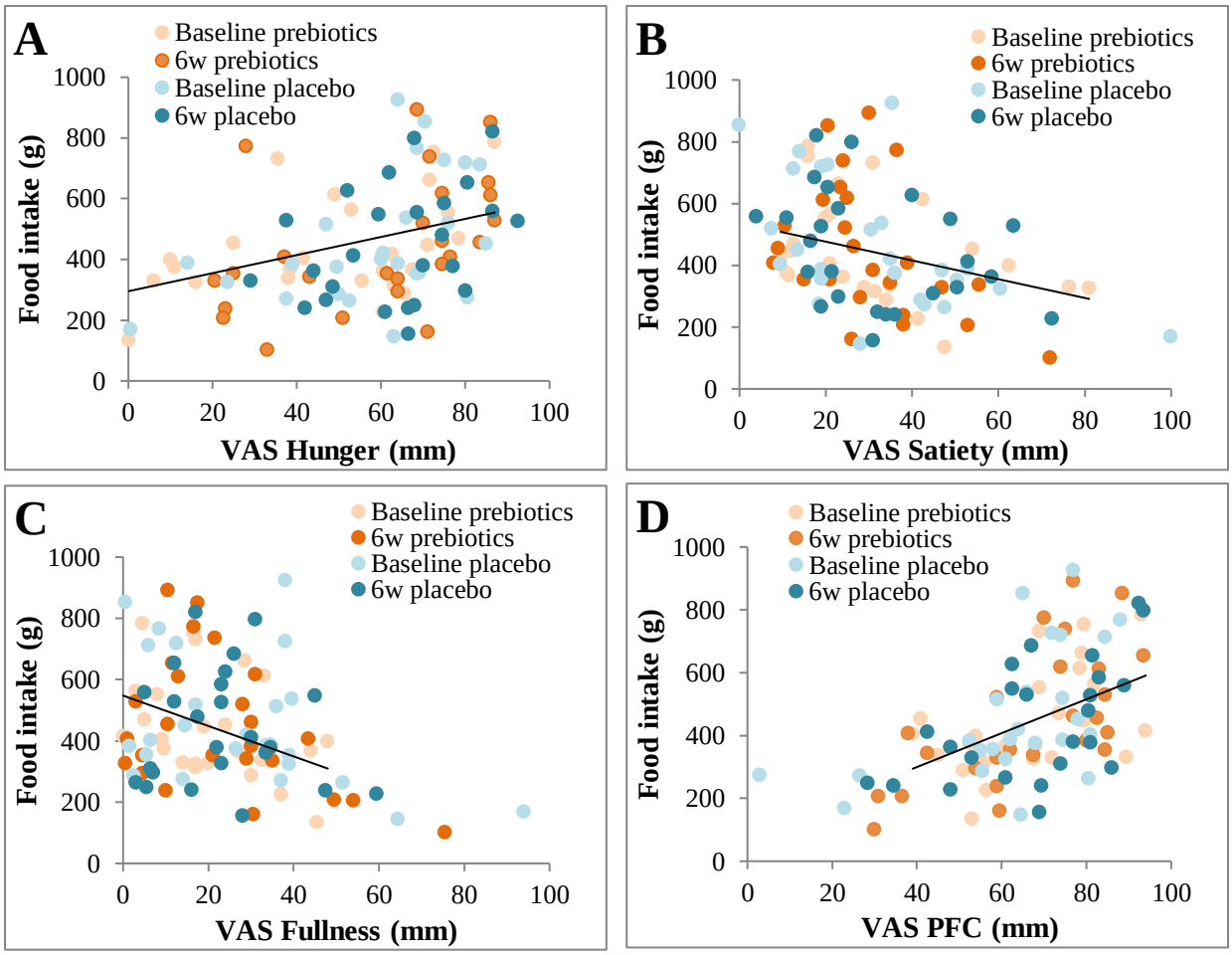

Figure 9. Correlations between food intake and subjective feeling of **(A)** hunger, **(B)** satiety, **(C)** fullness and **(D)** PFC are presented as scatter plots.
PFC: prospective food consumption; VAS: visual analogue scale; w: weeks.

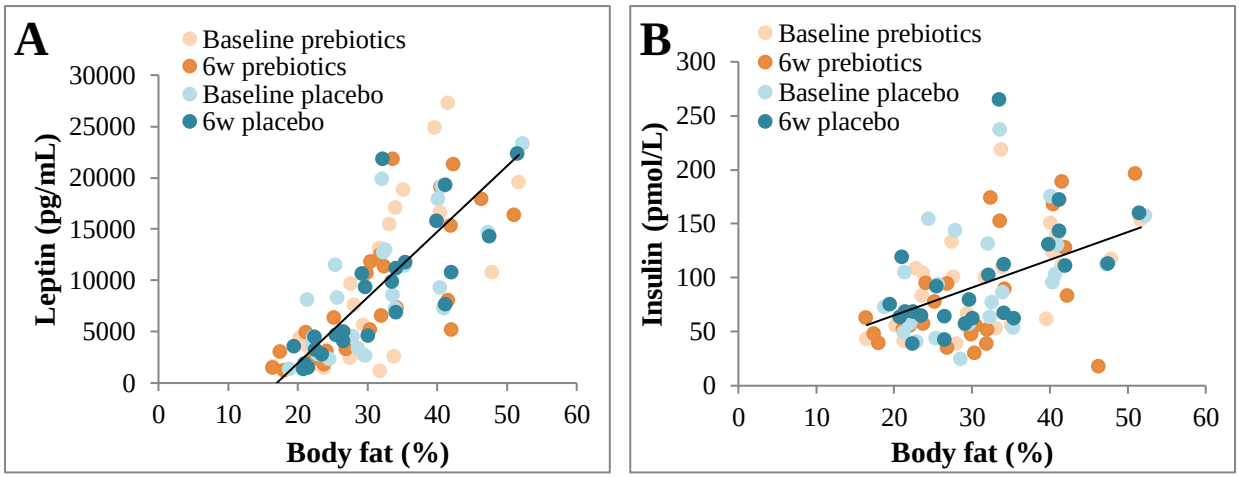

Figure 10. Correlations between body fat and **(A)** leptin and **(B)** insulin are presented as scatter plots.
W: weeks.

4 Discussion

In this study, we aimed to investigate the effect of prebiotic fiber supplement on subjective and objective appetite markers in people with T2DM. After 6w of treatment with both 16 g prebiotics and 16 g placebo divided by a 4w washout period, no effects of the supplement were found. Although we found some within treatment effects, there was no effect of a 6w supplement period with inulin and FOS on subjective feeling of appetite, food intake or objective appetite markers compared with placebo. There was a moderate inverse correlation between fullness and GLP-1, although no significant correlations between any other appetite related hormone and any of the appetite sensations or food intake were found. There were strong correlations between food intake and subjective feeling of hunger, satiety and PFC, and strong correlations between body fat and leptin and insulin.

Study design and methods

The Fiberdia study was a randomized controlled trial, which is considered to be the gold standard in providing evidence for causal treatment effects of a modifiable factor. The crossover design allows each participant to be its own control, thereby reducing the risk of confounding variables and between subject variability, allowing the sample size to be reduced. There are, however, some disadvantages with the crossover design. Firstly, if the treatment effect provided in the first test period had a long-lasting effect, carryover effects might be seen, and the impact of treatment in period two cannot be separated from treatment one (90). Previous research has shown that a 3w washout period provides sufficient time for gut microbiota to revert back to baseline (91). The minimum 4w washout period between the two study periods should therefore have been sufficient. However, when comparing baseline values, carryover effects were found for PYY for subjects who started with prebiotics. Statistically discovering carryover effects in small samples is difficult, and when discovering carryover effects for one variable, other related variables are also often affected. GLP-1 is secreted from the same type of enteroendocrine cells as PYY, and carryover effects may therefore also have occurred for GLP-1 without being discovered statistically. The results of PYY-, and possibly GLP-1 measurements, should therefore be interpreted with caution. Secondly, the participants have to be enrolled in the study over a longer period of time than in a parallel study. This might lead to a higher dropout rate, both due to the added burden on the participants and the higher possibility of unexpected events occurring during the study

period. Eleven of the participants in this study discontinued the intervention due to either serious illness, use of probiotics, antibiotics or insulin, or for other reasons declined to participate. A dropout rate of 25% was considered acceptable, though the dropout rate in this study was 30.6%. If the length of the study had been shorter, some of the participants who dropped out might have been able to complete. A parallel study would have reduced the length of the study; however, a parallel study would have required a greater sample size, and recruitment would have exceeded the time available for recruitment and data collection. To compensate for a higher risk of dropout, some measures to reduce the burden for the participants were made. These include covering travel expenses, economic compensation with 1000 kr per visit if they had to take the day off from work without pay, reminders by SMS with everything they had to do at certain time points, notice of next appointment by both mail, email and SMS, and the opportunity to contact study personnel by phone at all hours.

The daily dose of prebiotic fibers ingested during the intervention period was chosen based on previous studies. Most studies that have investigated the effect of prebiotic fibers on appetite related markers have used between 10 and 30 g/day (92, 93). However, one study showed that there is a need of higher doses (around 35 g/day) to get consistent effects on gut hormones, but that this dose might lead to side effects (94). Another study, investigating the effect of wheat fiber on gut peptides and SCFAs, found that it took 9-12 months before the rise in GLP-1 appeared in hyperinsulinemic subjects (20). Studies investigating change in microbiota have found significant increase in Bifidobacteria count after 7-14 days with ingestion of 5, 8, 10 and 20 g inulin and FOS daily, and a significant positive correlation between FOS dose ingested and fecal Bifidobacteria count (95, 96). They did, however, find a higher risk of adverse effects with 20 g/day compared with lower doses (95). With this in mind, we might have chosen a too low dose and too short intervention period to discover differences in appetite related hormones. However, 16 g/day have previously been shown to increase both subjective and objective satiety and decrease subjective and objective hunger, whilst avoiding adverse effects (26, 27). 16 g/day was therefore chosen as the daily dose for ingestion.

To consider if there was a specific time point during the MTT or *ad libitum* meal that gave significant differences in any of the variables, and to test if there was a different postprandial response before and after the study periods, all time points (0-180 mins) were tested for

change from baseline *within* the study periods and differences in change from baseline *between* the two study periods. Total AUC was also calculated and tested for differences within and between the study periods. AUC provides useful information about the total concentration of the appetite related hormones over time. Hence, many different statistical tests were performed. However, multiple statistical testing might lead to false positive inferences (type I errors) (97). For each statistical test performed, there is some probability (i.e. 5% when $p \leq 0.05$ is considered statistically significant) that an erroneous inference is made (97).

The purpose of this thesis was to investigate differences in change between the prebiotic- and placebo periods, not change within the study periods. There was no difference in treatment effect between prebiotic and placebo supplements for any of the appetite sensations, food intake or appetite hormones at any time point, except from PYY which showed a significant difference in change 60 min postprandially. There is a high risk of false positive inferences when investigating change within groups (98). False positive inferences due to multiple testing and testing changes within groups is probably what has caused the significant within group effects when analyzing subjective feeling of appetite and appetite related hormones, considering that the findings are not consistent. Thus, significant changes within the study periods are not emphasized.

Linear mixed model is a better statistical model for analyzing changes in repeated measures, such as in crossover studies. Advantages with linear mixed model is that it consists of both random and fixed effects, it allows both the between- and within-subject variance to differ among treatments, and it allows different numbers of measurements within each period (99). Linear mixed model was not performed in this thesis due to limited time and because it requires a high level of knowledge about statistics. However, it will be performed in the main study.

The statistical power, or ability to detect effects, of a study is directly tied to its sample size (100). The sample size of the Fiberdia study was calculated based on the relevant effect on GLP-1. Power calculations were not done based on relevant effects on subjective appetite markers, PYY, AG, leptin or insulin, and the sample size might not have given statistical power great enough to reveal differences between the prebiotic- and placebo period for these variables. In addition to this, most of the data was not normally distributed and statistical

analyzes were therefore performed using non-parametric tests. Non-parametric tests are more robust but have less power than parametric tests (97, 100).

Subjective appetite markers

VAS is a validated method to record subjective feelings of appetite and motivation to eat in humans (86-88). However, we experienced some challenges with using the VAS forms. The first four forms at each visit were filled out before the *ad libitum* lunch, and then at the exact time points 30, 60 and 90 min postprandially under controlled circumstances in a test room at the hospital, whereas the last form (180 min) was filled out outside of the hospital. Participants were reminded to fill out the 180 min form by SMS. However, it is uncertain whether the VAS form was filled out at the correct time point when filled out unmonitored. It might be suggested that the participants did not understand how important accuracy of time was when filling out the form. Filling out the VAS form regardless of time might have led to bias because the participants tried to remember how they felt instead of filling out exactly how they felt at the given time point. The VAS forms filled out 180 min postprandially might therefore be biased.

The participants were allowed to interact during the *ad libitum* meal. Subjects filling out forms in the test room together could therefore have influenced each other's answers.

Participants received nutritional drinks for breakfast after an overnight fast. Even though the nutritional drinks contained a substantial amount of energy, a 12 hour fast and only receiving liquid energy for breakfast is different from the participants' regular habits. Their normal appetite sensations might be influenced by falling out of their regular schedule.

Subjective feeling of appetite is also influenced by a number of external factors, such as physical activity, temperature, weather, menstrual cycle etc. on the test day (87). There is no way to distinguish between biologically day-to-day variations in appetite, and methodological variations (87). Day-to-day variations could have influenced appetite sensations differently on the different test days. All these factors might have led to biased records of appetite. However, hunger, satiety and PFC were strongly correlated with food intake, which provides some certainty that measurements of appetite sensations were valid.

To make the eating situation as similar as possible for all participants, all subjects ingested the *ad libitum* meal in company with other people, either other participants or study personnel. If only one subject was present at one of the visits, study personnel also ate the meal in company with the participant. Study personnel were always present during the meal

to remind the participants to weigh their food and fill in all the questions in the VAS forms. Ready-to-serve meals often have a more negative reputation than home-cooked meals, which are thought to be healthier and more tasteful. How the participants perceived the ready-to-serve meal might have influenced their food intake and also how the other participants in the room perceived the meal. Food intake might therefore be influenced by other participants' food intake, their attitude towards the meal and the feeling of being observed by other participants and health professionals (101).

Fjordland stopped the production of the first test meal in the middle of the study period. The dish used for the *ad libitum* meal was therefore switched to a meal with similar taste and content. For twenty-one subjects this was not an issue because they consumed the same meal at all four visits. Because they were their own controls, which meal they preferred did not affect food intake. For four of the participants, however, the *ad libitum* meal changed while they were enrolled in the study. They therefore consumed two different test meals during their participation in the study. Paired samples t-test showed that these four participants thought the second test meal (Chicken meat balls with vegetables and whole grain pasta) had significantly more aftertaste than the first meal. There were no differences in visual appearance, smell, taste or food intake between the two test meals. Because there was only n=4 participants that consumed two different test meals, we did not expect to find any statistical differences. However, the participants did not give the impression that they liked one better than the other. Still, we cannot rule out the possibility that preference for one of the meals may have influenced food intake at the different visits.

Previous research indicate that women have significantly higher spontaneous energy intake in their premenstrual phase (luteal phase) compared to the postmenstrual phase (follicular phase) (102). Some previous studies have included female participants at the same time of their menstrual cycle at every visit to avoid that hormonal differences affect food intake and appetite (103-105). This was not regulated in the present study due to practical reasons. Most women participating in this study were of postmenopausal age, and menstrual cycle should therefore not be an issue. However, for some of the participants, hormonal differences due to phase of menstrual cycle might have affected food intake and appetite sensations at the *ad libitum* lunch.

Appetite related hormones

AG, GLP-1, PYY and leptin were measured using a multiplex assay. A great advantage with this method is that it enables the simultaneous quantitation of several targets at once. However, using this method led to a substantial percentage of the samples being below the detection limit for AG (8.1%), GLP-1 (1.9%) and PYY (3.6%), but not for leptin. This led to a great number of the values being imputed as the lowest detectable value in the analyses, which may have reduced the strength of the results. The multiplex assays used here were based on antibodies binding to specific targets. If not well validated, there is a possibility of unspecific binding. This method for measuring AG, GLP-1, leptin and PYY was validated by the manufacturer (106). Their study showed that the multiplex assay had a high recovery rate (94-107%) and good precision for all metabolites. They also showed a strong correlation between active GLP-1 measured with the multiplex assay and ELISA (r=0.90) (106). In addition to this, our study showed a strong correlation between leptin and body fat, which indicates valid measurements of leptin using the multiplex assay. Both ELISA and Western blot are good alternatives to the multiplex assay. However, these methods can only analyze one target at a time and are therefore more expensive and time-consuming. For practical and economic reasons, the multiplex assay was chosen for analysis of appetite related hormones.

Our study found that the multiplex assay had an intra-assay CV of ≤ 20 % for AG, GLP-1 and PYY, and ≤ 15% for leptin. All blood samples from each individual participant were analyzed in the same assay. This means that differences of up to 15% for leptin and 20% for AG, GLP-1 and PYY found when investigating treatment effect might be due to variability in the assay or handling of the samples (pipetting etc.) rather than actual change in gut hormone concentrations. This could have contributed to the few significant within and between treatment effects that we found.

Values below the detection limit for appetite related hormones were imputed with the lowest detectable value when analyzing the data. When calculating the AUC for appetite related hormones, missing values were extrapolated if ≥3 values were present and there was a value at the time point before/after the missing value. Imputed values are not necessarily the true values, and by imputing values for missing data and values below the detection limit the results might be affected.

GLP-1, PYY and AG are well established as biomarkers for short-term appetite studies (107, 108). Leptin, however, is thought to be a poor biomarker for short-term appetite studies, because it is a long-term regulator of appetite and is not acutely influenced by food intake (107). Leptin was included in this study because it has been shown that SCFAs increases secretion of leptin (84). We did not expect to see postprandial changes in leptin levels but hypothesized that a 6w treatment period would be enough to see a change in leptin from baseline to after the prebiotic period.

In the samples used for insulin measurements, 3.5% were hemolyzed, whereas the hemolysis rate for samples used to measure appetite related hormones is unknown. Red blood cells contain insulin-degrading enzyme (109, 110). In a hemolyzed blood sample, insulin is degraded to multiple inactive insulin fragments, hence reducing the level of insulin in the sample. Wu *et al.* found that insulin concentration decreases with increasing percentage and exposure time of hemolysis. They also found that low temperature markedly reduced insulin degradation by insulin-degrading enzyme (110). Samples used for insulin analysis were centrifuged and cooled down after collection, which would have reduced insulin degradation in hemolyzed samples. Only one of the hemolyzed insulin samples was considered an outlier, and hemolyzed samples were therefore included in the analysis. Although most of the hemolyzed samples were not considered outliers, hemolysis might have affected the results. No studies have looked in to the effect of hemolysis on GLP-1-, AG-, PYY- or leptin concentrations using the multiplex assay, and it is uncertain how hemolysis might have affected these results. However, degradation of GLP-1 and AG was prevented by adding DPP-IV- and serine protease inhibitors before blood collection, and by putting the samples in the freezer immediately after collection.

Sixty percent of the participants used metformin. Metformin is shown to affect the prevalence of some bacterial species in the gut (111). Metformin can also enhance secretion of GLP-1 and PYY, which explains some of its glucose lowering effect (112). Because it performs some of its effects in the gut, metformin may lead to gut related adverse effects, like abdominal distension, nausea, flatulence, diarrhea and vomiting (112). In addition to this, the Summary of Product Characteristics for metformin states that it can cause abdominal pain and that it can affect appetite (113). Through these mechanisms, metformin might affect both subjective feeling of appetite, level of appetite related hormones and food intake. However, the participants were told to quit medications two days before the visits, and any gut related

side effects should therefore not be an issue. Furthermore, the participants could not change their medication or dosage during the study period. Because of the crossover design and the fact that all subjects were their own control, the effect of metformin on gut microbiota and appetite related hormones should not matter when comparing study periods.

Strengths and limitations

This present study has several strengths. Firstly, the longitudinal study design, with participants being their own controls. Secondly, compliance to the study was very good. Thirdly, both subjective and objective appetite markers were measured, both fasting and in response to a meal. This gave us the opportunity to compare any hypothetical differences between subjective feelings and actual biomarkers for appetite. Finally, as intended no significant weight change was found during the study period.

The study also presents some limitations. Firstly, due to the lack of valid measurement methods, the intake of prebiotic fibers from the regular diet was not controlled during the study period. A potential change in dietary intake of prebiotic fibers could therefore have happened without being noticed. However, dietary intake of fiber was measured by food frequency questionnaire both before and after the two intervention periods and no change was detected (Master thesis by Kristine Duus Molven, unpublished). Secondly, other dietary factors may also affect appetite. Diet was not regulated and may have varied between the intervention periods. Thirdly, greater sample size would have been desirable, to increase the statistical power. Finally, a carryover effect was seen in PYY, which means that our washout period might have been too short.

Discussion of results

No differences were found when investigating subjective appetite sensations. Most previous research have found reduced feeling of hunger (26, 27, 114, 115), increased feeling of satiety (26, 82, 115), increased feeling of fullness (115, 116), reduced feeling of PFC (26, 105, 116) and reduced food intake (25, 26, 28, 116) after treatment with inulin-type fructans. Our findings are conflicting to these studies, although some authors have found effects corresponding to ours in hunger (28, 104, 116), fullness (26, 104), PFC (104) and food intake (104, 105, 114, 115).

Correlations between appetite sensations and food intake were calculated using the average pre-lunch values for each of the appetite sensations, and average food intake of the four test

days. Correlation coefficients in the present study are stronger than what is found in studies validating the VAS forms for hunger (r_s=0.55 vs. r=0.32), satiety (r_s=-0.58 vs. r=-0.42) and PFC (r_s=0.71 vs. r=0.39), but not for fullness (87). This provides some certainty that appetite sensations were correctly measured.

There were no differences in appetite related hormones between the study periods, with the exception of a greater increase of PYY 60 min postprandially after the placebo- compared with prebiotic period. Previous studies have found reduced levels of ghrelin (28) and increased levels of GLP-1 (25, 27), PYY (25, 27, 28, 114) and insulin (117) after intake of inulin-type fructans in healthy, overweight, obese and prediabetic subjects, which are different from the present results. Even though conflicting from most studies, results from the present study do coincide with some previous research concerning GLP-1 (28, 116), PYY (116), leptin (116) and insulin (27, 114, 116). Some studies also found increased levels of ghrelin (116), and reduced levels of leptin (28) and insulin (28, 82) after supplementation with inulin-type fructans, which shows opposite results compared to our hypotheses. Different amounts and composition of inulin-type fructans, different duration and different study population in the previous studies might be reasons for the varying results.
There was a significant inverse correlation between GLP-1 and feeling of fullness, which is probably a random finding considering the suggested satiating effect of GLP-1. There were no correlations between AG, leptin, PYY or insulin and hunger, satiety, fullness, PFC or food intake. This suggests that the concentration of appetite related hormones did not necessarily contribute to how the subjects in this study perceived appetite sensations, and that the appetite related hormones did not predict food intake. However, appetite related hormones were measured during the MTT, whereas appetite sensations and food intake were measured approximately 3-3.5 hours later at the appetite test. The reason that we did not find correlations between subjective and objective appetite markers may therefore be because they were not measured at the same time.

PYY was analyzed in parallel samples in addition to paired samples because of the carryover effect. When analyzed in parallel groups, there were no differences in treatment effect between the prebiotic and placebo period. The analysis done in parallel samples have low power due to the small sample size, and differences in treatment effect may therefore not have been discovered. However, it confirms that the carryover effect may have affected the

results for PYY, and that the results from the analysis done in paired samples should not be emphasized.

When comparing levels of appetite related hormones in this study to those of healthy subjects, there seemed to be some differences. Fasting concentrations at baseline were in this study 64.5 pg/mL (P_{25}, P_{75}: 23.2, 154.3) for AG, 6.9 pg/mL (P_{25}, P_{75}: 2.7, 14.8) for GLP-1, 109.7 pg/mL (P_{25}, P_{75}: 48.6, 180.2) for total PYY and 7573 pg/mL (P_{25}, P_{75}: 2599, 15648) for leptin. Yau *et al.* found AG concentrations of 156.7 ± 77.3 pg/mL and leptin concentrations of 3542 ± 2525 pg/mL in healthy men measured with the same multiplex assay as in the present study (118). Douglas *et al.* found AG concentrations of 104 ± 58 pg/mL and total PYY concentrations of 103 ± 54 pg/mL in healthy, lean subjects (119). Plasma concentrations of AG seem to be lower, leptin concentrations higher, and PYY concentration relatively similar in our study compared with previous findings in healthy people.
The difference in leptin concentrations is probably caused by difference in body fat content. Most of the participants in the present study were overweight/obese whereas participants in the study by Yau *et al.* were normal weight. Leptin is secreted by adipose tissue, and leptin concentrations are strongly correlated with body fat content (37). Furthermore, overweight and obesity can lead to leptin resistance. Leptin resistance is shown by increased circulation leptin, which match our findings of increased leptin in the diabetic compared with the healthy subjects (120). High levels of leptin might also have diminished the desired effects of prebiotics on change in plasma leptin concentration.
One study found significant inverse correlations between AG and BMI and AG and waist circumference. They also found an inverse association between insulin resistance and AG (121). The lower AG levels in the present study can therefore partly be explained by overweight/obesity and insulin resistance.
Finding normal values for GLP-1 was difficult, because most studies have either analyzed total GLP-1 or do only present their data as figures or AUC.

Insulin secretion and response are severely altered in people with T2DM. Blood glucose is regulated by a feedback loop involving the pancreatic β-cells and insulin sensitive tissues, such as the skeletal muscle, adipose tissue and the liver. T2DM occurs when the insulin sensitive tissue no longer responds properly to insulin (insulin resistance) and β-cell function fails (122, 123). There may be interindividual variations in the participants' β-cell function

depending on e.g. time since diagnosis. Responses in insulin levels are therefore different than in healthy people, and the lack of effect of prebiotics on insulin can probably partly be explained by their diagnosis.

The effect of prebiotic fibers on increasing the number of health promoting bacteria in the gut relies on the initial composition of gut microbiota, with lower initial numbers leading to greater proportional increase in specific bacterial species (96, 124). People with T2DM have been shown to have a moderate degree of dysbiosis in the gut (24), and the effect of prebiotic fibers on altering microbiota composition might therefore be different than for healthy subjects (125). It has also been shown that there is great interindividual variability in how people respond to prebiotic fibers (125). It is unknown whether or not there is a greater number of nonresponders among people with T2DM compared with healthy subjects. However, if there is, this might be a possible explanation to why we did not find any effect of prebiotics on appetite markers.

People with T2DM are also known to have reduced effect of incretin hormones; both reduced GLP-1 response to meals and reduced insulinotropic potency of GLP-1 (53, 126, 127). The pathophysiologic mechanisms for the reduced incretin effect in patients with T2DM is unclear (126). However, dysbiosis and thereby reduced production of SCFAs, or resistance towards other hormones in addition to insulin, might be possible reasons. The reduced secretion and effect of incretin hormones might have diminished the desired effect of inulin and FOS on appetite markers, especially GLP-1.

As previously described, people with T2DM might have an altered secretion and function of insulin, leptin, ghrelin and GLP-1. This indicates that there is a resistance towards other appetite regulating hormones than just insulin in diabetic subjects. Whether or not this applies to PYY is unclear, although it has been shown that obese people have lower basal levels of PYY than healthy controls (128). Furthermore, reduced secretion in response to meals and other cues, or reduced peripheral sensitivity and physiological responses to the appetite regulating hormones, might explain why we did not see any effect of the prebiotics. This might also be some of the reason why we did not find an effect on subjective appetite markers. If the physiological signals do not work sufficiently, the appetite sensations and food intake might not reflect the body's nutritional state in the diabetic subjects.

This study is conducted in Norway, whereas previous studies that found effects of prebiotic fibers are conducted elsewhere. The intake of bread and other wholegrain products is high in Norway, and intake of fibers from diet might be higher than in other countries (129, 130). However, even though the intake of fibers is high in the Norwegian population, it does not necessarily translate to the intake of prebiotic fibers being high. There is no good database to estimate intake- and no established biomarker to measure intake of prebiotic fibers from diet. However, a potential higher dietary intake of prebiotic fibers before baseline may have diminished the potential effect of the prebiotic supplement.

Future perspectives

It is unclear why we do not find the same response to prebiotics in people with T2DM as in most studies in healthy people concerning appetite markers. The mechanisms behind this merit further research. Fecal samples have been collected in this study but have not yet been analyzed for microbiota composition. However, whether or not there is a larger number of nonresponders among participants in this study compared with participants in studies with healthy subjects will be investigated.

The comparison of appetite hormones in diabetic and healthy subjects done in the discussion of the results is only a comparison, and no tests are done to check for differences. Studying the effect of prebiotics in healthy subjects would give us data to statistically compare with diabetic subjects. As consumption of wholegrain products is high in Norway, it would also be interesting to study the effect of prebiotic fibers in a healthy Norwegian population. This could help us distinguish whether or not the absence of effect is due to the subjects having T2DM or if there are other factors playing a part, such as e.g. an already high intake of fibers.

5 Conclusion

In conclusion, we did not find any effects of prebiotic supplements on subjective appetite sensations, food intake or appetite related hormones in people with T2DM when compared with placebo. We suggest that this is caused by a resistance towards appetite related hormones in T2DM, similar to the insulin resistance seen in this population. Although more studies are needed to confirm our findings, reasons why effects are not found in people with T2DM need to be further investigated.

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Appendix I. Consent form.

Forespørsel om deltakelse i forskningsprosjektet

"Effekt av prebiotika (fiber) på tarmflora og hormoner som regulerer blodsukker og appetitt ved type 2 diabetes"

Bakgrunn og hensikt

Dette er et spørsmål til deg om å delta i en forskningsstudie for å se om det å spise en bestemt type fiber i seks uker, gir økning av sunne bakterier i tarmen. Vi vil undersøke om en eventuell økning i sunne bakterier i tarmen øker metthetsfølelsen og kroppens produksjon av insulin. Økt metthet vil over tid kunne føre til vektnedgang og gjøre at insulinet virker bedre blant personer som har type 2 diabetes. Sammen med bedret insulinproduksjon vil dette kunne bedre blodsukkerkontrollen ved type 2 diabetes. Studier på dyr og mennesker uten diabetes tyder på at fiber kan ha slike effekter, men vi vil undersøke om mennesker som har type 2 diabetes har samme effekt av å spise fiber. Ansvarlige for studien er tre kliniske ernæringsfysiologer og en lege, alle ansatt ved Oslo universitetssykehus, og alle undersøkelser vil bli gjort på Oslo universitetssykehus, Aker.

Hva innebærer studien?

Hver deltager får enten pulver med fiber eller pulver uten fiber (narrepulver/placebo) som de skal strø over maten to ganger daglig i seks uker. Så går det fire uker uten at man inntar pulver i det hele tatt. Deretter avslutter man med seks nye uker hvor man strør pulver med eller uten fiber over maten. Dersom man tok fiberpulver i de første seks ukene, avslutter man med narrepulver i de siste seks ukene og motsatt. Hverken studiedeltagerne eller de som driver prosjektet vet hvem som starter med fiberpulver og avslutter med narrepulver eller motsatt. Det avsløres ikke før prosjektet er slutt. Deltagerne skal ikke gjøre noen andre endringer i kosthold eller aktivitet i tiden studien pågår. Deltagerne vil bli kalt inn til en testdag før og etter hver av de to seksukersperiodene, altså totalt fire testdager:

Testdag på morgenen

Deltagerne kommer fastende til sykehuset og det gjøres en måltidstoleransetest. Dette foregår likt som ved glukosetoleransetest, bortsett fra at man inntar en næringsdrikke i stedet for ren sukkerløsning. Testen forgår slik: Det settes en veneflon (en liten slange) i den ene armen for å kunne ta flere blodprøver uten å måtte stikke flere ganger. Deretter drikker deltageren næringsdrikke (tilsvarende 75 g karbohydrater). Det tas blodprøver før man inntar næringsdrikken og tre ganger etterpå i løpet av to timer.

Testdag lunsj

Deltagerne forsyner seg så mye de ønsker fra en pastarett til lunsj. I forbindelse med måltidet registrerer man hvor mye man har spist og fyller ut et spørreskjema som måler appetitt, sult og metthet.

Vi vil også veie deltageren før denne undersøkelsen for å se om det er vektendring mellom studiestart og studieslutt.

Deltagelse i studien innebærer altså at man må møte opp på sykehuset fire morgener i løpet av en periode på ca fire måneder. I tillegg må deltageren svare på et spørreskjema om kosthold før og etter seksukersperiodene.

En viktig del av studien er å se på effekt av fiber på bakteriesammensetningen i tarmen. Deltagerne må derfor også samle to avføringsprøver i et lite prøveglass som de enten tar med på sykehuset eller sender i posten i ferdigfrankert konvolutt, én før og én etter hver av de to seksukersperiodene (totalt fire innsendinger).

Det vil gjøres en pustetest for å måle hvilke gasser som finnes i pusten før og etter fiberinntak. Dette gir også et bilde på hvilke bakterier man har i tarmen. Deltagerne gjør pustetesten i forbindelse med testdagene og pustetesten består av at man blåser gjentatte ganger i noen sekunder inn i et rør. Den tar til sammen ca 4 timer å gjennomføre og er ikke smertefull eller ubehagelig på noen måte.

Vedlegg A gir en mer detaljert beskrivelse av studien.

Mulige fordeler og ulemper

Fordeler

Ved å delta i denne studien får man en grundig vurdering av sin diabetesbehandling av helsepersonell som har god kunnskap om diabetes. Etter at studien er avsluttet vil man også få en muntlig tilbakemelding på resultatene av kostundersøkelsene man gjennomgår, hvis man ønsker det. En klinisk ernæringsfysiolog vil i tilfelle vurdere resultatene fra kostundersøkelsene opp mot anbefalt sammensetning av kosten ved diabetes og evt. gi forslag til endringer. Deltagerne får dekket alle utgifter i forbindelse med deltagelse i studien (som for eksempel reise med offentlig transport eller egen bil). I tillegg vil det ved studieslutt trekkes 5 tilfeldige deltagere som hver mottar et gavekort på kr 1000.

Ulemper

Blodprøvetaking/innsetting av veneflon kan være litt ubehagelig når en stikker gjennom huden, men det går fort over. Det kan benyttes lokalbedøvende krem som smøres på huden 1-2 timer før, dersom en deltager er svært engstelig. Fibertilskudd kan gi luftplager og ubehag i mage eller tarm, men dette er helt ufarlig og for noen går dette over etter noen uker. Vi vil registrere forekomsten av eventuelle bivirkninger og plager som måtte oppstå underveis i studien.

Hva skjer med prøvene og informasjonen om deg?

Prøvene tatt av deg og informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste. Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deg. All informasjon og prøvene som samles fra deltagerne skal slettes senest 31.12.2025. Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Frivillig deltakelse

Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn, trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling.

Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Om du nå sier ja til å delta, kan du senere trekke tilbake ditt samtykke uten at det påvirker din øvrige behandling. Dersom du senere ønsker å trekke deg eller har spørsmål om studien, kan du kontakte Eline Birkeland på telefon 980 24 193 eller Anne-Marie Aas 473 02 912.

Ytterligere informasjon om studien finnes i kapittel A – *utdypende forklaring av hva studien innebærer.*

Ytterligere informasjon om biobank, personvern og forsikring finnes i kapittel B – *Personvern, biobank, økonomi og forsikring.*

Samtykkeerklæring følger etter kapittel B.

Kapittel A- utdypende forklaring om hva studien innebærer

Kriterier for deltakelse

Du kan bli med i studien hvis du:

1. har type 2 diabetes
2. er over 18 år
3. har BMI: 25 - 40 kg / m^2 og
4. HbA1c: mellom 6,5 og 10,0 %

Du kan ikke bli med i studien dersom du:

1. bruker insulin
2. bruker GLP-1 -analoger (diabetesmedisin)
3. allerede har et høyt fiberinntak i kosten (over 25 g/ dag)
4. har endret vekt med mer enn 3 kg i løpet av de siste tre månedene
5. planlegger å følge en spesiell diett eller gå ned i vekt de neste 4 månedene
6. trener med høy intensitet mer enn 3,5 timer per uke
7. bruker antibiotika
8. tar tilskudd av prebiotika eller probiotika
9. er gravid
10. har en eller flere av følgende sykdommer: kreft, kroniske tarmsykdommer, irritabel tarmsyndrom, demens
11. ikke kan lese og skrive norsk
12. har høyt alkoholinntak (mer enn 3-4 alkoholenheter per dag)
13. bruker narkotika

Bakgrunnsinformasjon for studien

I løpet av det siste tiåret har flere studier vist en kobling mellom prebiotika som endrer tarmfloraens sammensetning, og insulinresistens og overvekt: Bruk av prebiotika har økt metthetsfølelse og forbedret glukosetoleranse og insulinutskillelse både i dyrestudier og humanforsøk. Noen studier peker på økt nivå av det insulinstimulerende hormonet GLP-1

som mulig mekanisme bak disse positive effektene av prebiotika og tarmflorapåvirkning. Det er imidlertid ingen studier som har undersøkt om prebiotika har disse effektene hos personer med type 2 diabetes, og om en evt. effekt gir seg utslag i bedre blodsukkerkontroll. Det er derfor interessant å studere om prebiotika (kostfiber) i form av kosttilskudd vil gi en endring i tarmfloraen og om den igjen vil øke insulinproduksjon og metthetsfølelse. Vi vil måle nivå av GLP-1 og PYY og andre relevante signalstoffer i blodet hos pasienter med type 2 diabetes for å se om det er en sammenheng mellom fiberinntak, tarmflora og disse signalstoffene i kroppen. Økt metthetsfølelse vil over tid kunne føre til vektnedgang og bedre insulinfølsomhet blant personer som har type 2 diabetes. Bedret insulinsekresjon og insulinfølsomhet vil kunne bedre blodsukkerkontroll ved type 2 diabetes.

Undersøkelser, blodprøver og annet deltageren må gjennom

Pusteprøver

Testen innebærer oppsamling av prøver av luft du puster ut ved faste tidspunkt. Prøvetagningen tar noen sekunder, er enkel å gjennomføre og uten ubehag. Etter inntak av lunsj tas prøver hvert kvarter første timen, deretter hver halvtime til testen er ferdig. Undersøkelsen varer omtrent i 4 timer.

Blodprøver:

Det tas fastende blodprøver før og etter de to seksukersperiodene du inntar pulver, og gjentatte ganger (0-180 min) etter en måltidstoleransetest.

Det vil også gjøres målinger av kortkjedete fettsyrer og Lipopolyosakkarider (LPS) i blod som et mål på endringer i tarmflora og tarmhelse.

Måltidstoleransetest:

- Det tas fastende blodprøver
- Du drikker næringsdrikke (tilsvarende 75 g karbohydrater)
- Næringsdrikken bør inntas i løpet av 5 minutter
- Du skal være i ro (sitte/ligge) under testen og skal ikke røyke
- Ny blodprøve tas nøyaktig 2 timer etter inntatt drikken

Avføringsprøver:

Du får med deg prøvetakingsutstyr hjem. Du gjennomfører prøvetaking ved hjelp av en avføringsoppsamler og tar en liten skje med avføring i et lite reagensglass (du får med deg instruksjon for prøvetagning). Du må levere prøvene innen 24 timer enten i ferdigfrankert konvolutt som du sender i vanlig post eller ta med prøven til sykehuset. Det er viktig at du rister prøveglasset etter at avføring er tilført prøveglasset slik at tilsetningsvæske og avføring blandes godt. Det er også viktig at **urin ikke** kommer opp i avføringsoppsamler

Tidsskjema – hva skjer og når skjer det?

0-3 mnd før studiestart

Justering av blodsukkersenkende behandling dersom behov. Deltageren skal ha uendret medikamentell behandling i 3 måneder før studiestart

To dager før DAG 1 i første seksukersperiode

- Du skal slutte med å ta medisiner for din diabetes (dersom du bruker det)

Én dag før:

- Én avføringsprøve tas hjemme
- Du skal ikke ha svært anstrengende fysisk aktivitet
- Du fyller ut spørreskjemaet om kosthold (FFQ)

12 timer før

- Faste

DAG 1 i første seksukersperiode = testdag

1. Møte fastende for blodprøvetaking
2. Måling av vekt
3. Levere utfylt spørreskjema om kosthold (FFQ) og avføringsprøve
4. Måltidstoleransetest med blodprøver
5. Testmåltid (pastarett) med pustetest og utfylling av spørreskjema om appetitt
6. Utdeling av pulver med eller uten fiber for seks uker

———————————

De neste seks ukene

- Første uken inntas én pose pulver hver dag
- De neste fem ukene inntas én pose pulver to ganger hver dag

———————————

To dager før SISTE DAG i første seksukersperiode

- Du skal slutte med å ta medisiner for din diabetes (dersom du bruker det)

Én dag før

- Én avføringsprøve tas hjemme
- Du skal ikke ha svært anstrengende fysisk aktivitet
- Du fyller ut spørreskjemaet om kosthold (FFQ)

12 timer før:

- Faste

SISTE DAG i første seksukersperiode = testdag

1. Møte fastende for blodprøvetaking

2. Måling av vekt
3. Levere utfylt spørreskjema om kosthold (FFQ) og avføringsprøve
4. Måltidstoleransetest med blodprøver
5. Testmåltid (pastarett) med pustetest og utfylling av spørreskjema om appetitt

——————————

Fire uker uten pulver

——————————

To dager før DAG 1 i andre seksukersperiode

- Du skal slutte med å ta medisiner for din diabetes (dersom du bruker det)

Én dag før:

- Én avføringsprøve tas hjemme
- Du skal ikke ha svært anstrengende fysisk aktivitet
- Du fyller ut spørreskjemaet om kosthold (FFQ)

12 timer før

- Faste

DAG 1 i andre seksukersperiode = testdag

1. Møte fastende for blodprøvetaking
2. Måling av vekt
3. Levere utfylt spørreskjema om kosthold (FFQ) og avføringsprøve
4. Måltidstoleransetest med blodprøver
5. Testmåltid (pastarett) med pustetest og utfylling av spørreskjema om appetitt
6. Utdeling av pulver med eller uten fiber for seks uker

——————————

De neste seks ukene

- Første uken inntas én pose pulver hver dag
- De neste fem ukene inntas én pose pulver to ganger hver dag

——————————

To dager før SISTE DAG i andre seksukersperiode

- Du skal slutte med å ta medisiner for din diabetes (dersom du bruker det)

Én dag før

- Én avføringsprøve tas hjemme
- Du skal ikke ha svært anstrengende fysisk aktivitet
- Du fyller ut spørreskjemaet om kosthold (FFQ)

12 timer før:

- Faste

SISTE DAG i andre seksukersperiode = testdag

1. Møte fastende for blodprøvetaking
2. Måling av vekt
3. Levere utfylt spørreskjema om kosthold (FFQ) og avføringsprøve
4. Måltidstoleransetest med blodprøver
5. Testmåltid (pastarett) med pustetest og utfylling av spørreskjema om appetitt

Mulige fordeler

Du vil ikke ha noen spesielle fordeler av studien, men erfaringer fra studien vil senere kunne hjelpe andre med samme diagnose. Funnene fra studien har også betydning for hvilke kostanbefalinger som gis i forhold til inntak fiber, både type og mengde, for personer som har type 2 diabetes.

Mulige bivirkninger:

Det er ingen risiko å delta i studien bortsett fra det ubehag det kan påføre.

Mulige ubehag/ulemper

Fiber

Bruk av fiber kan medføre ubehag i form av luft i magen. Vi vil registrere forekomsten av mage og tarm bivirkninger som en del av total vurderingen av behandlingen. De positive effektene av fiberbehandling som vi ønsker å undersøke vil sannsynligvis oppveie evt. ubehag/ulemper ved behandlingen og blodprøvetakning.

Blodprøvetaking

kan være litt ubehagelig når en stikker gjennom huden, men det går fort over. Noen kan bli uvel under prøvetakingen. Det vil derfor benyttes lokalbedøvende krem som smøres på huden 1-2 timer før dersom en deltager er svært engstelig.

Avføringsprøver

Det kan oppleves litt ubehag å samle opp for noen av pasienter. Deltagerne får med seg instruksjon for prøvetakning og tar med prøvetakningsutstyr hjem. De kommer ikke i fysisk kontakt med avføring, men tar en liten prøve fra et oppsamlingsnett. Det benyttes en spesialspatel til dette som føres direkte inn i et lukket prøveglass. Oppsamlingsnettet skyldes ned sammen med avføringen i toalettet. Prøveglasset legges i ferdigfrankert konvolutt og sendes inne 24 timer.

Tidskrevende

Det tar noe tid å delta i studien; det tar ca 1 time å fylle ut spørreskjemaene du skal svar på om matinntak og eventuelle bivirkninger ved inntak av fiber, og du må stille opp 4 formiddager i løpet av ca 4 måneder til tester med måltidstest og lunsjtest. Prosjektet skal vurderes og tilrås av REK før prosjektet sette i gang.

Pasientens/studiedeltagerens ansvar

- Stiller opp til undersøkelsene og prøvetaking
- Faste før blodprøver, glukosetoleransetest og pusteprøver
- Registrere mulige bivirkninger
- Registrere matvarefrekvensskjema (FFQ)
- Registrere appetitt, sult og metthet og sult på testdagene

Du vil bli opplyst så raskt som mulig dersom ny informasjon blir tilgjengelig som kan påvirke din villighet til å delta i studien.

Du skal bli opplyst dersom det kommer opp om mulige beslutninger/situasjoner som gjør at din deltagelse i studien kan bli avsluttet tidligere enn planlagt.

Eventuell kompensasjon til og dekning av utgifter til deltakerne

Det vil blitt gitt kompensasjon for utgifter i forbindelse med deltagelse i studien (utgifter til reise med offentlig transport eller egen bil). Deltagelse i studien innebærer dessuten ekstra oppfølging av helsepersonell med spesialkompestanse innen diabetes og deltagerne får tilbakemelding på kostregistrering ved fullført studie.

Ved studieslutt trekkes 5 tilfeldige deltagere som hver mottar et gavekort på kr 1000.

Kapittel B - Personvern, biobank, økonomi og forsikring

Personvern

Opplysninger som registreres om deg er ditt kosthold, ernæringsstatus (vekt, høyde), medisiner, blod-, avførings- og pusteprøver. For å oppnå hensikten med studiet vil det være nødvendig å hente opplysninger fra journalen din. Opplysninger i din journal er underlagt taushetsplikt i henhold til Helsepersonelloven, og bare de som trenger å se den i forbindelse med undersøkelse og behandling har tilgang til den. Du har krav på innsyn i alle opplysninger vi har om deg. I tillegg har du rett til å få supplert, rettet eller slettet informasjon som er feil.

Biobank

Prøvene som blir tatt og informasjonen utledet av dette materialet vil bli lagret i en forskningsbiobank ved Oslo universitetssykehus. Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyseresultater inngår i biobanken. Førsteamanuensis Anne-Marie Aas er ansvarlig for biobanken. Biobanken planlegges å vare til 2025. Etter dette vil materiale og opplysninger bli ødelagt etter interne retningslinjer.

Utlevering av blodprøver og opplysninger til andre
Bortsett fra blodprøver vil alle analysene bli gjort i laboratorier i Norge. Hvis du sier ja til å delta i studien, gir du også ditt samtykke til at dine blodprøver avidentifiserte opplysninger utleveres til Novo Nordisk Foundation center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark.

Rett til innsyn og sletting av opplysninger om deg og sletting av prøver
Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Økonomi
Studien og biobanken er finansiert gjennom forskningsmidler fra Helse og Rehabilitering for en stipendiat. Vi har også fått ekstra midler for analyser fra Diabetesforbundets og Mills DAs forskningsfond. Det er ingen ytelser til sykehus, forskningsleder, eller forskningsstiftelser. Det er også ingen interessekonflikter.

Forsikring
Pasientskadeloven gjelder ved deltagelse i studien

Informasjon om utfallet av studien
Resultatene vil offentliggjøres i internasjonale tidsskrift. Du har rett på innsyn i alle opplysninger og resultatet vi har om deg.

Samtykke til deltakelse i studien

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)

Appendix II. Nutritional content per 100 ml nutritional drinks.

	Fresubin 2 kcal Drink vanilla	**Fresubin Jucy Drink apple**
Energy (kcal)	200	150
Protein (g)	10	4
Fat (g)	7.8	0
Carbohydrate (g)	22.5	33.5
Fiber (g)	0	0

Nutritional content in nutritional drinks used for the meal tolerance test.
Participants received 200 ml of the Fresubin 2 kcal Drink with vanilla taste and 100 ml of the Fresubin Jucy Drink with apple taste at every visit.

Appendix III. Nutritional contents per 100 g Fjordland meals**.**

	Pasta with meatballs and tomato sauce	**Chicken meat balls with vegetables and whole grain pasta**
Energy (kJ)	478	453
Energy (kcal)	114	108
Fat (g)	2.5	2.9
- Saturated fat (g)	1.2	0.8
Protein (g)	4.9	5.6
Carbohydrate (g)	17.3	13.9
- Sugars (g)	4.8	4.5
Fiber (g)	1.1	2

Nutritional content in Fjordland meals used for *ad libitum* lunch. Pasta with meatballs and tomato sauce was the first meal used. Chicken meat balls with vegetables and whole grain pasta replaced pasta with meat balls and tomato sauce meal after it went out of production.

Appendix IV. VAS form for measuring appetite.

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Skjema for VAS –appetitt

Studiedeltager nr: □□ **Deltagers initialer:**□□□

Dagens dato: □□/□□-□□□□ **TESTDAG:** □

Tid 0 ☐ **Tid 30 min (akkurat spist)** ☐

Klokkeslett Tid 0= □□:□□

Tid 60 min ☐

Tid 90 min ☐

Tid 180 min ☐
Fylles ut klokka:
□□:□□

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Instruksjon:
De følgende spørsmålene skal besvares ved å angi i hvor stor eller liten grad du opplever det som spørres om.
Sett en liten strek /ett merke på linjen der du føler passer best.
Helt i venstre ende av linjen betyr *lavest* tenkelig grad.
Helt i høyre ende av linjen betyr *høyest* tenkelig grad.

Hvor sulten føler du deg?

Jeg er ikke sulten i det hele tatt

Jeg har aldri noensinne vært mer sulten

Oslo universitetssykehus
Effekt av prebiotika ved diabetes type 2

Hvor mett føler du deg?

Jeg er skrubbsulten

Jeg er stappmett

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Hvor full føler du magen din er?

Magen er ikke full i det hele tatt

Helt full

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Hvor mye tror du at du orker å spise?

Ingenting

Mye

Appendix V. VAS form for measuring palatability.

Oslo universitetssykehus
Effekt av prebiotika ved diabetes type 2

Skjema for VAS – smak

Studiedeltager nr:□□ **Deltagers initialer:□□□**

Dagens dato: □□/□□-□□□□ **TESTDAG: □**

Fylles ut ved tid 30 min (= like etter måltidet)

Instruksjon:
De følgende spørsmålene skal besvares ved å angi i hvor stor eller liten grad du opplever det som spørres om.
Sett en liten strek /ett merke på linjen der du føler passer best.
Helt i venstre ende av linjen betyr lavest tenkelig grad.
Helt i høyre ende av linjen betyr høyest tenkelig grad.

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Matrettens innbydenhet/utseende

God Dårlig

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Matrettens lukt

God Dårlig

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Matrettens smak

God Dårlig

Oslo universitetssykehus

Effekt av prebiotika ved diabetes type 2

Matrettens ettersmak

Mye Ingen

Appendix VI. Flow-chart of recruitment process.