The relationship between relevant biomarkers and perceived hunger during the postprandial period in healthy young adults: The PoMet intervention study

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Abstract

Background: A lot of research has been conducted on the field of appetite regulation. However, less in known about the impact of the metabolic switch and the increasing ketone production on subjective feelings of hunger and satiety during postprandial adaptation in healthy, young men and woman.

Objective: Focusing on dynamic changes in plasma glucose, insulin concentrations and ketones, the primary objective was to explore the relationship between relevant biomarkers and perceived hunger, satiety, and desire for food during postprandial adaption in healthy, young adults.

Methods: Thirty-six healthy adults aged 20-30 years with a body mass index of 22-27 kg/m^2, was served a standardized breakfast meal before fasting for 24 hours. Venous blood samples were drawn at 13 time points during the postprandial period. The samples were collected frequently during the first four hours, then every other hour for the subsequent eight hours up to 12 hours. Additionally, one last blood sample was conducted the following morning, 24 hours after the meal. Biomarker concentrations was measured using a targeted metabolomics approach, (body composition was measured by air displacement plethysmography using a BodPod), and data on perceived hunger, satiety and desire for food was assessed through a set of 14 visual analogue scales, filled out by the participant at each blood sampling.

Results: Overall, we observed an expected increase in concentrations of glucose and insulin immediately after food consumption, peaking at approximately one hour, before returning to baseline values. For the ketones, the lowest concentrations were observed during the early postprandial period, followed by a gradual increased throughout the intervention. Moderate to strong correlations were observed between the biomarkers and the parameters of perceived hunger, satiety, and desire for food.

Conclusion: In this study, glucose and insulin were associated with less hunger and more satiety, while the opposite was observed for the ketones. However, more research is needed in order to account for day-to-day variations and strengthen the validity of the results. Future research should involve longer study duration with repetitive interventions to provide a stronger basis to explore the potential impact of the metabolic switch and ketone production

on perceived hunger and satiety, and further supplement the findings of the current study with measurements of established hormones related to hunger and satiety.

Keywords: fasting, postprandial, metabolism, hunger, satiety, ketones, glucose, insulin

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ACAC	Acetoacetate
ADF	Alternate day fasting
AIPE	Accuracy-in-parameter-estimation
ATP	Adenosine triphosphate
CCK	Cholecystokinin
CR	Calorie restriction
DAG	Diacylglycerol
E%	Energy percentages
FA	Fatty acid
FFA	Free fatty acid
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GC-MS/MS	Gas chromatograph - Tandem mass spectrometry
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
IF	Intermittent fasting
MAG	Monoacylglycerol
NEFA	Non-esterified fatty acid
PEPCK	Phosphoenolpyruvate carboxykinase
PYY3-36	Peptide tyrosine tyrosine
SOP	Standard operating procedure
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TG	Triglyceride
VLDL	Very low-density lipoprotein
WHO	World Health Organization
βHB	Beta-hydroxybutyrate

1. Introduction

Obesity represents a substantial health problem with increasing prevalence, affecting men and women of all ages. With a burgeoning population of more than 1 billion obese individuals worldwide, obesity represents a global epidemic (1). The condition is associated with several non-communicable diseases of modern societies, including hypertension, type II diabetes mellitus, insulin resistance, and atherosclerosis, and some of its co-morbidities consistently rank within the World Health Organization (WHO) list on top 10 causes of death (1-3).

Under optimal circumstances, satiety should be tightly connected with feelings of hunger and satiation in a way that gives a close match between food consumption and energy expenditure. However, the current obesity epidemic indicates a strong deviation from such optimal correlations and the widespread, unfavorable development suggests an unmet clinical need for obesity treatment. In context of this pervasive health problem, it is important to fully understand the regulatory mechanisms behind the human appetite, particularly those controlling the adjustments of energy intake to energy needs.

A prolonged restriction in energy intake has consistently been found to reduce body weight and extend healthy life span in humans, leading to the consensus that calorie restriction is the main approach for weight loss (4). Evidence from human clinical trials suggest that calorie restrictions (CR) in overweight and obese individuals not only lead to reduction in body mass, but also serve a number of beneficial physiological effects, including improvement of insulin-sensitivity, mitochondrial function and several cardiovascular risk factors (5). A modest weight loss of 5–10% of the initial body weight, in combination with lifestyle modifications, has been shown to provide significant clinical health benefits (6). Hence, the treatment of overweight and obesity have wider objectives than weight loss alone, additionally focusing on risk reduction, management of co-morbidities and overall improvement of life quality and health.

Numerous international guidelines on adult obesity management have been published in Europe, Australia, and the US. Current European recommendations for weight loss therapy in overweight and obese individuals largely follow the recommendations in selected major

national and international guidelines, all recommending a daily energy deficit of 500-750 kcal/day for sustainable weight loss (6-10).

Unfortunately, even though it is considered common knowledge that a reduction of the overall calorie intake in combination with regular exercise will help optimize body weight, the problem of poor compliance in traditional weight loss programs is well known (11). Findings from obesity intervention trials over the past several decades shows an exceedingly high recidivism rate and suggests that most individuals have difficulty sustaining daily CR in the long term (12). With obesity being defined as a chronic disease, lifelong weight management is crucial for reduction in health risks, and hence appropriate goals of weight management should include prevention of weight regain (6).

1.1 Intermittent fasting

In recent years, intermittent fasting (IF) has gained attention as an alternative strategy for reducing energy intake (13). The term intermittent fasting describes a variety of eating patterns that involves partially or entirely abstaining from food for a set amount of time on a recurring basis, where the fasting time intervals range from 12 hours up to several days (5). As the main requirement of a dietary weight loss plan is that the total energy intake should be less than the energy expenditure, a fasting dietary regime can be an alternative approach to the continuous daily energy restriction, as long as the total energy expenditure exceeds the number of calories consumed.

IF integrates a myriad of modalities, varying in calorie allowances, duration of the fasting period, and the number of hours or days in between each fasting window. On a general basis, IF can be categorized into six main strategies: time-restricted eating, alternate day fasting (ADF), alternate day modified fasting, twice-weekly fasting (the 5:2 diet), modified periodic fasting, and religious fasting (14). Common to all IF-strategies is that they only require CR for limited time periods, which serves as a contrast to the daily CR regime and may facilitate adherence.

Compared to traditional CR paradigms, the IF strategy has shown promise in delivering similar benefits regarding both weight loss and risk markers for metabolic disease (15-17). An umbrella review of 11 meta-analyses of randomized clinical trials describing outcomes associated with intermittent fasting on obesity-related health outcomes suggest that IF may

have a beneficial role in improving anthropometric and cardiometabolic outcomes, including body weight, fat mass, fasting plasma glucose, fasting insulin, insulin resistance, low-density lipoprotein (LDL) cholesterol, total cholesterol, triglycerides, and blood pressure (18). A small-scale study by Varady et al. reports that ADF is effective for weight reduction and improve heart/cardiometabolic health in both normal weight and overweight individuals (19). Over a 12-week period, the researchers found that the 32 participants lost an average of 5.2 kg. Similarly, Harvie and colleagues looked at the effects of the 5:2 fasting style in 23 overweight women, and found that the participants on an average lost 4.8 percent of their body weight over a 5-week period (20). A third study, conducted in 107 overweight and obese women over a 6-month period, reported that continuous CR and restricting calories twice weekly both led to similar weight loss, and found that the 5:2 eating pattern improved insulin sensitivity and reduced insulin levels among participants (21).

Although multiple studies have shown that placing time restrictions on feeding trigger similar physiological pathways as continuous CR, and that none of the interventions produces superior changes in body weight when compared to the other, reviews suggest that ADF may increase insulin sensitivity more than daily calorie restricted diets based on similar weight loss and indicate a favorably impact of the ADF regime on hormones involved in the regulation of hunger and satiety (11).

1.2 Energy metabolism

As foods are consumed by humans, glucose, fatty acids, and amino acids, are released through digestion and absorption of the three energy-containing macronutrients carbohydrates, fats, and proteins, respectively (22). These metabolites provide substrates for both immediate and future energy needs. In humans, glucose and fatty acids (FAs) are the preferential fuel substrate for energy metabolism, with the relative importance of each dependent upon the availability of food (23). Post absorption, breakdown products of the energy-containing nutrients, as well as vitamins, minerals, and water are transferred to the bloodstream, and transported through the lymphatic and portal vein circulation system to the liver (24).

The liver has long been seen as an essential organ for energy metabolism. By acting as a hub, it metabolically connects various tissues, including skeletal muscle and adipose tissue, and hence governs the energy metabolism in the body (25). In the postprandial state, when carbohydrates are abundant, insulin concentrations are elevated, and the utilization of glucose

predominates (23). Concurrently, there is a flow of free fatty acids (FFAs) from the liver to adipose tissue. In this state, the liver is responsible for the conversion of glucose into glycogen and lipids, which provide metabolic fuels during fasting conditions. In the transition from postprandial to fasting state, insulin concentrations drop, leading to increased release of FFAs from adipocytes (23). When entering the fasting state, glucose is produced and secreted by the liver through both glycogenolysis and gluconeogenesis to ensure a steady supply of circulating blood glucose, and FFAs converted into ketone bodies to provide supplemental metabolic fuels for extrahepatic tissues (25). The metabolic pathways of main interest in this thesis are glucose and lipid metabolism, which are described in more detail in the following paragraphs.

1.2.1.1 Glucose metabolism

In the postprandial state, blood glucose from the portal vein enters hepatocytes via facilitated transport by the plasma membrane glucose transporter GLUT2 (24). Once inside the hepatocyte, glucose is phosphorylated by glucokinase to generate glucose 6-phosphate (G6P), ensuring the permanence of this metabolite inside the cell (26). A reduction in intracellular glucose concentrations leads to further increase in glucose uptake (25). From this step, G6P is either metabolized into pyruvate through glycolysis, condensed into glycogen through glycogenesis, or metabolized via the pentose phosphate pathway to generate NADPH required for lipogenesis and biosynthesis of other bioactive molecules (24).

1.2.1.2 Glycolysis

Glycolysis is dominant in the postprandial state in which glucose is abundant. The breakdown of glucose to pyruvate through glycolysis serves two main purposes; to produce adenosine triphosphate (ATP) and to produce precursors for synthesis of fatty acids or amino acids (27). Thus, pyruvate is either completely oxidized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria to generate ATP or used to synthesize fatty acids through *de novo* lipogenesis (25).

1.2.1.3 Glycogenesis

In times of nutritional sufficiency, glycogen, the branched polymer of glucose, acts as an energy store in liver and muscle tissue (28). The process of glycogen synthesis from newly ingested carbohydrates is termed glycogenesis and occurs when blood glucose levels are sufficiently high to allow storage of glucose for subsequent utilization. In glycogenesis,

glycogen synthase joins glucose to a core of glycogen via α-1,4 linkage, and as the number of joined glucose molecules reach 10-12 in each branch, a new branch is made via α-1,6 linkage by branching enzyme, resulting in a complex glycogen molecule for storage (27).

1.2.1.4 Glycogenolysis

Under fasting conditions, the liver acts as a superior organ that cooperates with different extrahepatic tissues to provide sufficient energy to the body and plays a central role in producing glucose via glycogenolysis and gluconeogenesis to maintain healthy blood glucose levels/euglycemia (24). During the first several hours of food deprivation, and until hepatic glycogen stores are depleted, the liver produces and releases glucose mainly through glycogenolysis (5). Glycogenolysis are considered the principal route of glycogen utilization and can be described as the process in which stored glycogen is hydrolyzed by glycogen phosphorylase to generate glucose, supporting fuel needs of different extrahepatic tissues throughout the body (25).

Glycogenolysis occurs via two distinct pathways. The first pathway involves synchronized enzyme action by glycogen phosphorylase and glycogen debranching enzyme in the cytosol, whereas the second pathway concern lysosomal degradation by alpha-glucosidase. However, less than 15% of glycogen degradation happens in lysosomes (29). Corresponding to cytosolic degradation, glycogen phosphorylase and glycogen debranching enzyme catalyze the release of glucose-1-phosphate (G1P) respectively from glycogen branches at the alpha-1,4 and alpha-1,6 positions. G1P is then converted into G6P, which is further hydrolyzed into glucose and inorganic phosphate by glucose-6-phosphatase (30). Asides for providing energy, glycogenolysis generate precursors for oxidative reactions of the pentose phosphate pathway, yielding NADPH required for lipogenesis (27).

1.2.1.5 Gluconeogenesis

During prolonged fasting, when liver glycogen stores are depleted, hepatic gluconeogenesis is the primary source of glucose production. Gluconeogenesis refers to the metabolic pathway by which glucose is synthesized from non-carbohydrate precursors (24). The precursors of gluconeogenesis are either generated in the liver or delivered from extrahepatic tissues through the circulation (25). The principal gluconeogenic substrates include pyruvate (product of glycolysis), lactate (produced from pyruvate), glycerol (product of triglyceride

breakdown), and certain amino acids (primarily alanine), and with propionate making a minor contribution (31).

Lactate is oxidized by lactate dehydrogenase to generate pyruvate. The process of gluconeogenesis starts with the carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase in the mitochondria (24). Oxaloacetate is required to be used in the cytoplasm, but is not diffusible to the mitochondrial membrane, and hence needs to be reduced to malate before exported into the cytoplasm where it is oxidized to regenerate oxaloacetate (27). After multiple biochemical reactions, involving the two key rate-limiting enzymes phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase among others, oxaloacetate is converted into G6P, transported into the endoplasmic reticulum, and dephosphorylated by glucose-6-phosphatase to release glucose (25). Glycerol in turn, released from adipose tissue through lipolysis, is phosphorylated to generate glycerate-3-phosphate, a precursor for gluconeogenesis, whereas amino acids, released through protein degradation in muscle tissue, are converted to α-ketoacids via deamination reactions (25). The α-ketoacids are metabolized to TCA cycle intermediates (e.g., oxaloacetate and pyruvate) which serve as precursors for gluconeogenesis (27).

1.2.2 Lipid metabolism

In the postprandial state, dietary fat is digested in the small intestine and further absorbed into enterocytes. Inside enterocytes, fatty acids are resynthesized into triacylglycerol (TAG) before secreted into the gut lymphatic system as chylomicrons (24). Chylomicrons arrive at the liver through the circulation, and undergoes lipolysis mediated by lipoprotein lipase to release non esterified fatty acids (NEFAs) (25). NEFAs enter hepatocytes in proportion to their blood concentration, by diffusion or mediated by fatty acid transport protein or fatty acid translocase (32). Furthermore, in abundance of carbohydrates, glucose is not only utilized as the main metabolic fuel and stored in the form of glycogen, but also converted into fatty acids through de novo lipogenesis in the liver (25).

1.2.2.1 Lipogenesis

Lipogenesis encompasses the processes of fatty acid synthesis from nonlipid precursors and subsequent synthesis of more complex lipid structures (33). The synthesis of fatty acids requires acetyl-CoA, malonyl-CoA, and NADPH (22). Pyruvate, hydrolyzed from glucose through glycolysis, is imported into the mitochondria and metabolized to generate acetyl-CoA

(25). Acetyl-CoA feeds the TCA cycle, where it is combined with oxaloacetate to form citrate, exported into the cytoplasm, and split back into acetyl-CoA and oxaloacetate (34). Oxaloacetate is then reduced to malate and further converted into pyruvate, releasing NADPH, before pyruvate is transported back into the mitochondria where it is carboxylated to oxaloacetate which drives continuous citrate synthesis (25).

In the cytoplasm, de novo lipogenesis starts with the conversion of citrate to acetyl-CoA, which is then carboxylated to malonyl-CoA, involving several reactions (35). Both NADPH and malonyl-CoA are used as precursors to synthesize palmitic acid by fatty acid synthase. From this step, palmitic acid is modified by elongases and desaturases in the endoplasmic reticulum to generate a variety of different FAs (36). Hepatocytes also obtain FAs from the bloodstream, which are absorbed from food digestion in the gastrointestinal (GI) tract (24). On a typical western diet in which FAs is abundant, the uptake of TAGs from the vasculature is by far the more important source of FAs to the liver (24).

Free fatty acids, produced through de novo lipogenesis or delivered to hepatocytes through the bloodstream, are incorporated into more complex lipid structures in one of three different means: incorporated into phospholipids, esterified with glycerol 3-phosphate to generate TAG, or esterified with cholesterol to produce cholesterol esters (36). Phospholipids serves as an essential component of cell membranes, bile particles, lipid droplets, and very low-density lipoprotein (VLDL) particles, whereas TAG and cholesterol esters are secreted into the bloodstream as VLDL or stored in the liver as part of lipid droplets (25).

1.2.2.2 Lipolysis

In addition to glucose produced via glycogenolysis and gluconeogenesis, the human body is dependent on supplemental energy substrates to provide sufficient energy in times of food deprivation. Hence, the fasting state promotes lipolysis in adipose tissue. Lipolysis refers to the metabolic process through which TAGs in adipocytes are broken down into their constituent molecules glycerol and FFAs via hydrolysis (37). During times of energy deprivation, adipose tissue is stimulated by hormonal and biochemical signals to shift towards greater net rates of lipolysis as lipolytic products serve as the main metabolic fuels in the fasted state (38).

The first step of lipolysis is the conversion of TAG into diacylglycerols (DAGs) and FFAs by adipose triglyceride lipase, before DAGs are further hydrolyzed by hormone-sensitive lipase, generating monoacylglycerols (MAGs) and FFAs as the second step (24). The last step of TAG hydrolysis involves monoglyceride lipase, resulting in the production of glycerol and a third FFA (37). Succeeding lipolysis, FFAs and glycerol are released into the vasculature and transported to the liver to produce energy through β-oxidation and ketogenesis (25).

1.2.2.3 Ketogenesis

Ketogenesis can be defined as the metabolic pathway in which ketone bodies are produces through breakdown of fatty acids in the mitochondria of liver cells (39). In the body of healthy humans, there is a continual but small production of ketones to be used for energy. In times of fasting, when carbohydrate stores are remarkably decreased and circulating fatty acid concentration are elevated, there is an upregulation of the ketogenic pathway, resulting in an increased production of ketone bodies that brings the body in a state of ketosis (40).

Once entered the liver cells, FFAs are transformed by fatty acyl CoA synthetase into fatty acyl CoA, which are susceptible to β-oxidation (41). Every cycle of β-oxidation generates one molecule of acetyl-CoA, in addition to one molecule each of FADH and NADH, apart from the very last cycle which produces two molecules of acetyl-CoA from even chained fatty acids, and one acetyl-CoA and one propionyl-CoA from odd chained fatty acids (30).

The process of ketogenesis primarily occurs in the mitochondria of hepatocytes, starting with the combination of two acetyl-CoA molecules into acetoacetyl-CoA by thiolase. From this step, HMG-CoA synthase adds another molecule of acetyl-CoA to form beta-hydroxy-beta-methylglutaryl-CoA, which in turn is metabolized to acetoacetate (AcAc) by HMG-CoA lyase (42). In times of further metabolization, AcAc can either be converted by beta-hydroxybutyrate dehydrogenase generating beta-hydroxybutyrate (βHB), or irreversibly decarboxylated via non-enzymatic decarboxylation into the third ketone body acetone (39).

The three ketone bodies, AcAc, βHB, and acetone, are in turn secreted into the bloodstream to provide an alternative energy source and sustain the function of brain and muscle cells during extended periods of fasting (25). βHB is the main plasma ketone body, typically present in about five times the concentrations of AcAc (41). The ratio of βHB to AcAc is determined by a reversible redox reaction, which is further related to the NAD+/NADH-ratio in

mitochondria of hepatocytes (43). Once the ketones reach extrahepatic tissues, they are metabolized through ketolysis to acetyl-CoA, which enters the TCA cycle to generate ATP (5). Acetone, however, does not convert back to acetyl-CoA, and is either exhaled or excreted through the urine (39).

In regards of nutritional ketosis, the βHB range has been suggested to be 500–3000 μmol/L (44), but after weeks of fasting, a ceiling in humans has been reported at approximately 6000–7000 μmol/L (45). For a more complete assessment of ketosis, plasma concentrations of both βHB and AcAc can be summed as total ketones. In the postprandial state, the total plasma concentration of ketone bodies exhibit circadian oscillations between approximately 100 and 250 μmol/L in healthy humans, providing about 5% of the body's total energy requirement (42). Following a fasting period of 24 hours, ketone body levels typically rise to 1000 μmol/L, further increasing to 5000–7000 μmol/L during prolonged periods of food deprivation. Ketone bodies provide about 20% of total energy requirements in the 24-hour fasting period and yields more than two thirds of the brain's energy requirement during prolonged fasting (42, 46). However, as βHB is dominating at increasing ketosis, and further due to the unstable nature of AcAc, the assessment of ketosis often relies solely on βHB in both clinical and scientific settings (44).

1.2.3 The metabolic switch

The liver has eminent flexibility in selecting between different metabolic fuels. During prolonged fasting when glycogen stores in hepatocytes are depleted, an alternative energy source is required, leading to the onset of the metabolic switch. The metabolic switch can be defined as the point of negative energy balance expressed through a shift from glucose to lipid metabolism, including the alteration from lipid synthesis and fat storage to fat mobilization in the form of FFAs and fatty-acid derived ketones (5). The timepoint for the metabolic switch is depending on the size of the liver glycogen store at the outset of the fasting period, as well as the energy expenditure during the fast. However, the switch occurs when hepatic glycogen stores are exhausted and the production of fatty acids and glycerol are increased trough accelerated adipose tissue lipolysis, typically beyond 12 hours after cessation of food consumption (5).

1.2.4 Regulating aspects of energy metabolism

Hepatic energy metabolism is tightly controlled by numerous transcription factors and coregulators, and multiple hormonal, neuronal and nutrient signals have been identified to affect the metabolic switch between the postprandial and fasted state. The activity of these nuclear proteins is regulated by metabolic hormones such as insulin and glucagon, which dynamically regulates the different pathways involved in hepatic energy metabolism in order to meet systemic metabolic demands.

In the postprandial state, the metabolic profile is predominantly regulated by insulin. Increasing levels of circulation blood glucose, fatty acids, and amino acids, triggers the pancreatic β cells to secret insulin (14). The hormone has multiple functions regarding metabolic regulation. The binding of insulin to a tyrosine kinase receptor on cell membranes triggers a complex series of events that stimulates glycogen synthase, acetylation of glycogen phosphorylase, and the expression of insulin-regulated glucose transporter GLUT-4 (responsible for insulin-stimulated glucose uptake into muscle cells and adipocytes) and glucokinase, hence increasing glycogen synthesis, suppressing glycogenolysis, promoting glucose uptake in extrahepatic tissue, and indirectly increasing glucose uptake in hepatocytes by facilitating phosphorylation of glucose to G6P (25). G6P in turn inhibits glycogenolysis and stimulates glycogen synthesis, consequently further increasing liver glycogen levels (25). Furthermore, insulin downregulate PEPCK gene expression and inhibits fructose-1,6-biphosphatase, the key rate-limiting steps of hepatic gluconeogenesis, and stimulates hepatic lipogenesis (47). Additionally, the availability of lipogenic precursors is further increased by insulin through glycolytic stimulation (25).

During the postabsorptive state, the hormone glucagon is secreted from pancreatic alpha cells (25). Glucagon counteracts insulin action concerning enzymes involved in glucose and lipid metabolism, thereby decreasing the synthesis of glycogen, increasing glycogenolysis, increasing gluconeogenesis by upregulating the expression of PEPCK, and increasing ketogenesis by causing a more notable breakdown of FFAs, thus increasing the amount available for the ketogenic pathway (39). In addition to glucagon, growth hormone, cortisol, and epinephrine are grouped together as counter-regulatory hormones, sharing an oppositional action toward insulin (47).

The central nervous system regulates energy metabolism in the liver both directly and indirectly, by controlling secretion of various metabolic hormones via the sympathetic and the parasympathetic nervous system (25). Through signals received from central and peripheral glucoreceptors, the hypothalamus regulates hormonal secretion in response to circulating blood glucose concentrations, to maintain this close to constant levels of around 4-5 mmol/L (24). As glycemia rise, the parasympathetic system is activated whereas the sympathetic system is inhibited, leading to increased insulin secretion. Contrarily, a glycemic drop produces the opposite response by inhibiting the secretion of insulin and promoting secretion of the counter-regulatory hormones. Hence, a close interaction of neuroendocrine mechanisms and hepatic autoregulation in response to serum glucose concentration, ensures the maintenance of euglycemia.

1.3 Appetite regulation

1.3.1 Early theories of appetite control

The biochemical basis for hunger and satiety have been subject of debate and extensive research for many decades. Early theoretical approaches to the regulation of appetite, food consumption and body weight are based on three main theories: the glucostatic, the aminostatic, and the lipostatic model, each focusing on the bodily status of one macronutrient (48, 49). In the mid 1950s, Jean Mayer postulated that the rise in glycemia after food consumption was the critical factor underlying satiety. According to Mayer's glucostatic hypothesis, a rise in blood glucose levels leads to an increased feeling of satiety whereas small declines have the opposite effect, triggering meal initiation (50). The theory posits that variations in blood glucose concentrations are detected by glucosensitive neurons in the brain that influence energy intake in a depletion-repletion manner (49, 50). However, the impact of carbohydrates on satiety somehow depends on the presence of both soluble and insoluble fiber, which has been shown to enhance satiety sensations, rather than the general availability of carbohydrates (51). Furthermore, contrary to the notion that hyperglycemia is the critical factor inducing satiety, later research has reported improved sensations of satiety, decreased energy intake and delayed onset of hunger following the intake of foods and meals with low glycemic index that induce modest rises in blood glucose levels (52). Additionally, despite the fact that fluctuations in blood glucose concentrations correlate well with food intake, the model of depletion and repletion do not describe the coordination of energy intake with expenditure that results in the evolutionary stability of adipose tissues in the long term (49).

Aware of this, Mayer emphasized that his glucostatic hypothesis account for the short-term regulation of food intake and suggested a lipostatic mechanism to account for the regulation of energy balance and body weight in the long term (53).

The lipostatic model, first described by Gordon Kennedy in 1953, links appetite to the amount of fat mass in the body (49, 54). This theory posits that adipocytes generates circulating signals proportional to the amount of stored body fat, which is passed to the brain and translated into effects on energy intake and energy expenditure to maintain homeostasis and compensate for potential discrepancies from a given setpoint (54). Kennedy proposed that high concentrations of the signal in question corresponded to excess body fat and lead to reduced intake to lower the amount of stored body fat. Contradictorily, low concentrations where assumed to be relative to depleted fat stores and lead to increased intake and decreased expenditure to bring the body into positive energy balance (54). In 1994, more than 40 years after the initial proposition, the discovery of leptin by Zhang and colleagues provided strong molecular basis for such a feedback system (48). The realization that leptin is secreted by adipose tissue to inform the brain of the current body adiposity, appeared to prove the authenticity of the lipostatic model and gave rise to a large and rapidly growing literature supporting the theory that food intake is controlled within a lipostatic system for energy homeostasis (49). A homeostatic regulation of the body fat mass could be a potential explanation why returns to the pre-dieting body weight typically occurs following attempts at weight loss.

In contrast to the theories on glycemia and adiposity, the aminostatic theory holds that circulating amino acids have the critical role in defining satiety. In 1956, Mellinkoff and colleagues proposed that aminos acids and their metabolites was the regulating parameter of satiety sensations (52). According to this view, the intake of protein, which elevates serum amino acid concentrations, induce satiety, and reduce food intake in humans. Evidence supporting such a nutrient-specific hierarchy of satiating power has accumulated over the past decades, with protein showing the highest potency compared to the other macronutrients (55).

1.3.2 Current situation on appetite control

In the field of appetite regulation, there has been devoted a lot of research to identify critical signals triggering and inhibiting food intake behavior, and evidence in support of all the above-mentioned theories, both clinical and experimental, has accumulated. Rather than

mutually exclusive options, these approaches seem to offer complementary perspectives regarding food intake control, and the balance of energy intake and energy expenditure seems to be regulated through a complex physiological system comprising afferent as well as efferent stimuli. There is now considerable consensus that specific areas in the brain integrates both signals of satiety and signals of long-term energy status to produce a coordinated response to the change in nutritional status after ingestion of a food or beverage, and that these signals are important regulators of food intake and energy balance in humans (56).

However, although appetite is primarily based on physiological demands, food intake in the modern society is strongly dependent on the environment and lifestyle. Food consumption can occur for multiple reasons, including social factors, opportunity, habits, and time of day, and hence, the onset and termination of eating are not solely tied to immediate energy needs and the replenishment of depleted energy substrates (57). Thus, in later years, it has become clear that regulating mechanisms of appetite is a result of several complex processes involving physiological as well as environmental, social, cognitive, and sensory factors.

The current situation on appetite control involves an overall consensus that appetite is primarily dependent on the interoceptive sense of whether energy levels are adequate (58). When food is present in the GI tract or available energy (e.g., blood glucose or lipid availability) are above a certain threshold value, satiety signals overrule signals of hunger resulting in a low feeding drive. Contrarily, hunger signals are generated in times of food deprivation or when energy levels are perceived as inadequate, further leading to the stimulation of food intake (58, 59). Hence, hunger is expected to be maximal prior to the onset of a meal. As food is ingested, hunger progressively decreases, paralleled by a rise in satiation. At the point of maximum satiation, hunger is absent, and satiety is at its highest (59). Over time, satiety drops, and the feeling of hunger gradually rises, preparing the body for the next meal.

The onset of satiety involves messages from mechano- and chemoreceptors in the oral cavity and GI tract, as well as release of gut peptides in response to a meal (56). Sensations of appetite and satiety in humans are processed in hypothalamic centers within the brain, and the nucleus of the hypothalamus serves as an important regulatory region for food intake behavior and energy homoeostasis (60). The endocrine and central nervous system is connected

through a bidirectional network of signaling pathways, the brain-gut axis, which controls eating behavior through biochemical signaling via hormones in the GI tract (61). Several endocrine signals that regulate appetite neuronal circuitry have been identified (58). The GI hormones can be divided into two main groups: stimulators of appetite, predominantly ghrelin, and stimulators of satiety, including leptin, glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and peptide tyrosine tyrosine (PYY3-36) (61). The appetite stimulating peptides elicit meal initiation by increasing hunger feelings, whereas satiety stimulators signal the brain to promote meal cessation by suppressing hunger (61). Next to these GI hormones, glucose and insulin play an important role in food intake behavior (61).

Insulin is positively correlated with body weight and circulates in the bloodstream at levels proportional to peripheral energy stores (62). The pancreatic hormone is released from beta cells in response to food intake and crosses the blood-brain barrier to act as a satiety stimulating signal within the brain (49). Hence, in interaction with plasma leptin, insulin fulfills many of the requirements as a hypothalamic energy regulator, providing essential negative feedback signals to the central nervous system, coupled with catabolic circuits and proportional to the amount of stored body fat (62). However, as insulin levels are highly sensitive to the immediate effects of food consumption, its primary physiological function is control of glucose homoeostasis rather than body weight regulation (56).

2. Objectives

As the body alters between the postprandial and fasting state, the energy metabolism is subject to constant change, switching from glucose to fatty acids and fatty acid derived ketones as the preferential energy substrate. The various metabolic pathways are tightly regulated in order to meet the dynamic requirements, and appetite signals, comprising a number of overlapping systems, is a crucial part of this regulatory aspect. The main objective of the present thesis is to investigate how concentrations of blood glucose, insulin, and ketones fluctuates during 24 hours of fasting, and to explore how perceived hunger and satiety changes during postprandial adaptation, and how this relates to circulating concentrations of the above-mentioned metabolites.

Clearly, a lot of research has been conducted on the field of appetite regulation. However, less in known about the impact of ketone bodies on subjective feelings of hunger and human food intake behavior. In this regard, this thesis will look closer into the mechanisms of appetite regulation and perceived hunger during a longer period of food deprivation, focusing of the potential impact of the metabolic switch and the onset of ketone body production in the postprandial period in healthy, young men and woman.

3. Methodology

The present thesis is based on The Postprandial Metabolism (PoMet) intervention study, which is a collaboration between Haukeland University Hospital and Mohn Nutrition Research Laboratory at the University of Bergen. The aim of the PoMet study is to extend the knowledge on the dynamics of postprandial metabolism in human. By monitoring circulating concentrations of a broad range of biomarkers during 24 hours after a standardized meal, the study will provide time-resolved biomarker-specific data during postprandial adaptation. Accordingly, the main objective is to describe the dynamics of the biomarkers, focusing on B-vitamin status, amino acids and metabolites related to one-carbon metabolism. The study was registered at Clinicaltrials.gov (NCT04989478) on 04.08.2021.

3.1 Sample size

The planning of sample size for a contemplated study can be addressed in various ways, predominantly using one out of two main strategies. The most common approach involves calculating the statistical power of the planned study (63). Following this method, the sample size is calculated based on which effect you want to detect, by estimating the number of participants needed to get a p-value below a chosen cutoff (commonly 0.05), with a given probability (statistical power, commonly at least 80%). The second strategy is to calculate the number of participants based on precision, by aiming to obtain a desired width of a confidence interval for the targeted effect, referred to as an accuracy-in-parameter-estimation (AIPE) approach (64). The latter approach is used in the PoMet study, as the main purpose is to describe the changes in metabolites with a certain precision rather than to achieve statistical significance for specific comparisons.

Our goal was to measure at least 80% of the biomarker concentrations within a margin of error of 10%, corresponding to a geometric standard error (gSE) < 1.05. To estimate the required sample size, available data from the HuMet study on 132 metabolites across 56 different time points was utilized (freely available from http://metabolomics.helmholtz-muenchen.de/humet/). The geometric standard deviation (gSD) was calculated for all 7392 estimates. The calculations gave an observed median gSD of 1.24, with an 80th percentile of 1.32, meaning that 80% of the obtained gSD's were below 1.32.

$$\mathbf{1)}\ gSE = gSD^{\frac{1}{\sqrt{n}}}$$

Assuming that 80% of our measurements would have a gSD <1.32, **equation 1** was rearranged and solved for n to achieve a gSE < 1.05 (corresponding to a margin of error < 10%), and a sample size of 32 was found to achieve the desired precision level. With the expectance of up to 10% dropout due to adverse events following fasting blood sampling or difficulties drawing blood from a venous catheter, a total of 36 participants was required to achieve the goal of collecting complete data in 32 participants.

3.2 Recruitment and participants

Criteria for inclusion and exclusion are described in **Table 1**. The aim was to recruit 36 healthy, normal-weight individuals aged 20-30, both male (n=18) and female (n=18). To reach the specific target group, the recruitment was focused on posters at local universities, student houses, gyms, and grocery stores, as well as snowball sampling and advertisements on social media. Subjects who were interested in participating in the study were asked to fill out a web-based questionnaire. All subjects were contacted by one of the researchers for a pre-screening by phone.

Table 1 Inclusion- and exclusion criteria for participation in the PoMet-study

Inclusion criteria	Exclusion criteria
Aged 20-30 years (birth years 1991-2001)	Acute or chronic disease during the last three years
Self-reported BMI 22-27 kg/m^2 at phone screening	Food allergies interfering with the standardized breakfast meal
	Use of any prescription medications except contraceptives
	Smoking or regular use of other nicotine-containing products
	Pregnancy or breastfeeding during the last three months
	Weight change (> 5%) during the last three months

BMI; Body Mass Index

3.3 Preparations in advance of attendance

To maximize the comparability between subjects and homogenize physiological and metabolic conditions, participants were asked to not use any dietary supplements the week before attendance, and to abstain from alcohol and avoid strenuous physical activity during the last 24 hours before start-up. To standardize blood samples and anthropometric measurements at baseline, participants were instructed to eat a meal similar to the standardized breakfast intervention at approximately 8:00 PM the evening before the study

visit, and to not consume anything but water after completion of the evening meal and until attendance. The evening meal should consist of one glass of orange juice and three slices of bread, which of two with butter, white cheese, and cucumber, and one with butter and jam (preferably jam with little or no added sugar). Participants were asked to bring the total volume of morning urine at attendance, and to be well hydrated to facilitate the insertion of a venous catheter.

3.4 Study visit

The course of the study visit is illustrated in **Figure 1**. The participants attended the study center at 07:30 AM. With the last meal at 8:00 PM the evening before, all participants attended the study center in an approximate 12 hour overnight fasting state. At start up, informed consent was signed before participants were screened to ensure that they were eligible for participation. Further, anthropometric measurements were conducted, and BMI was calculated. Due to influencing factors when measuring body mass, including hydration status, impact from clothing and the use of different scales, small deviations from the self-reported BMI inclusion criteria was accepted.

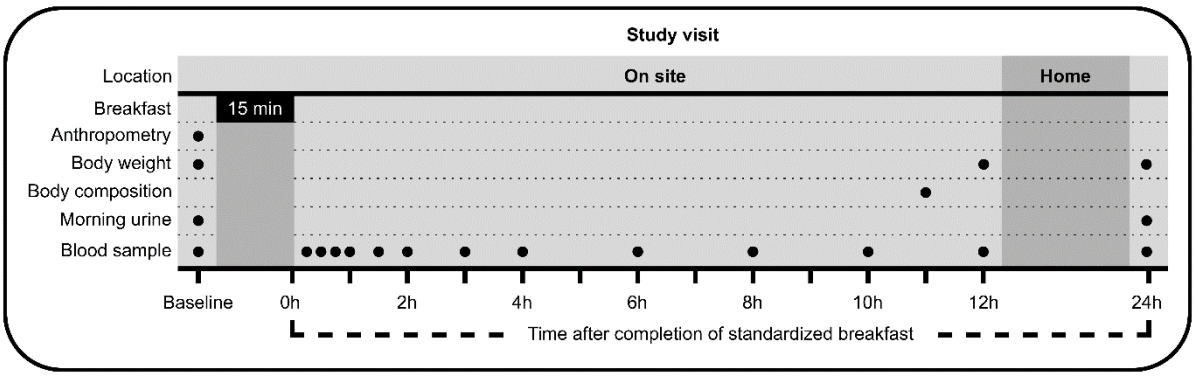


Figure 1 Schematic overview of the study visit in The PoMet study.

A venous catheter was installed in the elbow cavity of the participants after the conduction of anthropometric measures, following a fasting blood sample. After collection of the first blood sample, a standardized breakfast meal was served. Participants was instructed to use precisely 15 minutes to consume all parts of the repast, including a glass of juice. After completing the standardized meal, participants were not allowed to consume any food or beverages for the ensuing 24 hours.

Venous blood samples were drawn, and capillary blood glucose was measured, at 13 specified time points after the meal, starting 15 minutes post consumption (**Figure 1**). The first four hours following the meal, blood samples were drawn frequently (15, 30, 45, 60, 90, 120, 180, and 240 minutes after completion of the meal). For the subsequent eight hours, the samples were collected every other hour up to twelve hours. Data on perceived hunger, satiety and desire for food was assessed through a set of 14 visual analogue scales (VAS), filled out by the participant at each blood sampling. VAS were collected shortly after completion, so that participants did not have access to their previous ratings when answering the evaluative questions. 10 hours into the fasting period, body composition was measured using a BodPod (COSMED). Participants had free access to water during the study visit, apart from the last two hours before the body composition analysis.

After 12 hours of fasting, bodyweight was measured under the same circumstances as in the morning, before the participants were free to leave the study center to spend the night at home. The participants were instructed to not engage in strenuous physical activity and to consume nothing but water until returning to the study center for a final 24-hour fasting blood sample the next morning. A third measurement of bodyweight was conducted at 24 hours. Both mornings, morning urine was collected at home before attendance and brought to the study center by the participant, either in a clean and dense jar or in a suitable container delivered by the research group prior to the study visit.

3.4.1 The breakfast meal

According to recommendations from the Norwegian Directorate of Health, a breakfast meal should constitute 20-30% of an individual's daily energy needs, which is estimated to be 2150 and 2600 kcals per day for inactive females and males, respectively (65). Further, in regards of macronutrients composition, the Norwegian Directorate of Health recommends that carbohydrates accounts for 45-60 energy percentages (E%), whereas fats for 25-40 E%, and protein for 10-20 E% (65). The breakfast meal consumed by the participants was composed to be in line with the above-mentioned recommendations, and to imitate a regular Norwegian breakfast in terms of food choices. A detailed description of the foods and macronutrient content in the breakfast meal is provided in **Table 2.** The meal contained 513 kcal, with 46.3 E% from carbohydrates, 35.9 E% from fats, 16 E% from protein, and 1.9 E% from dietary fiber. Hence, the breakfast meal posed approximately 20% of estimated daily energy

requirements for male participants and 24% of estimated daily energy requirements for female participants. The SOP for preparations of the standardized meal is described in **appendix 1**.

Table 2 The breakfast meal in the PoMet-study

Food	Amount, g	Energy, kcal	Fat, g	Carbohydrate, g	Dietary fiber, g	Protein, g
Wheat bread	90	213	4.7	34.5	4.0	6.2
Butter	15	81	9	0.1	0	0.1
Light cheese	40	107	6.4	0	0	12.4
Jam	20	26	0	6.2	0.3	0.1
Orange juice	200	82	0.4	18.2	0.2	1.4
Cucumber	36	4	0	0.4	0.3	0.3
Total	**401**	**513**	**20.5**	**59.4**	**4.8**	**20.5**
Energy %	**-**	**-**	**35.9**	**46.3**	**1.9**	**16**

3.5 Data collection

Data were collected from participants from August to November during autumn 2021. All biological samples, as well as all information of relevance, were obtained during one 12-hour visit at the study center for each participant, except from one final blood sample collected the following morning.

3.5.1 Anthropometric measures

Anthropometric measures were carried out at the same time of day and by the same project staff for all participants. The height measurement was conducted using a Seca Stadiometer, model 217. The height was measured in light clothing without shoes to the nearest 0.1 cm. Participants were asked to have their feet gathered, legs straight, shoulders relaxed, arms along the side, and to look straight ahead to keep the head in the Frankfurt horizontal plane. The back of the head, shoulder blades, butt and heals was up-close to the vertical measurement pole during the procedure. The measurement was conducted once, with maximal inspiration, and the measurement was documented directly in the participant folder. The standard operating procedure (SOP) for height measurement is described in **appendix 2.**

Weight measurements at baseline, and after 12 and 24 hours fasting were conducted using a Seca scale, model 877. In prior to the measurement, participants were instructed to remove shoes and heavy clothing, and to empty all pockets. The measurement was carried out once, and the measurement was documented in the participant folder directly. The SOP for weight measurements is described in **appendix 3.**

The measurement of waist circumference was conducted using a Seca 203 measuring tape. A small pen mark was made at the lower rib and upper point of the hip bone of the participant. The measurement was conducted horizontally in the middle of the two pen marks, directly to the skin. Participants were instructed to breath normally. The measurement was performed on exhalation, and rounded to the nearest 0.1 cm, while the participant's arms hung down along the sides. The measurement was carried out three times for each participant, and the average value of the three measurements was documented in the personal folder. The SOP for measurement of waist circumference is described in **appendix 4.**

3.5.2 Blood glucose measurements

Prior to every blood sampling, blood glucose was measured using a HemoCue Hb 201+ Analyzer. Capillary blood from the participants middles and ring fingers were used for sampling. At the first two timepoints, participants were asked to wash their hands before the samples was collected. A lancet was used to puncture the finger, and the first drop of blood was wiped away. The next drop was collected with a HemoCue Hb 201 microcuvette in one continuous process, before the filled cuvette was directly placed in the cuvette holder on the HemoCue Hb 201+ Analyzer for analysis. The result was manually entered into the participant folder.

3.5.3 Blood sampling

Blood samples were drawn at 14 time points in total, of which 13 during the 12-hour study visit and 1 collected the subsequent morning after 24 hours of fasting. Blood samples were drawn through a venous catheter placed in the elbow cavity of the participants and was carried out by trained personnel. At each time point, the venous catheter was flushed with 2,5 ml saline solution before the 3-way port was established and a waste tube was used to draw 2,5 ml from the catheter prior to blood sampling. Further, the designated tubes were filled. A total of 11.5 ml blood was drawn at each time point, distributed into two serum tubes à 5 and 3.5 ml (BD Vacutainer® SST™ II) and one EDTA tube à 3 ml (Vacuette® K2EDTA). After the final tube was filled, the venous catheter was flushed with 10 ml saline solution. For measurement of HbA1c and safety biomarkers, an additional 6 ml EDTA blood was collected at baseline, along with an additional 3 ml EDTA blood at the 24-hour timepoint. The aim was to draw all samples within ±2 minutes from the designated sampling timepoint. If at any time the flow through the venous catheter was discontinued, the participant and the on-site staff

made a shared decision whether a new catheter should be placed, or normal venous blood samples should be taken, depending on the number of blood samples remaining and the participants preferences. Regardless of function, the venous catheter was removed for all participants after the 10-hour fasting blood sample to allow measurement of body composition. Thus, the 12- and 24-hour fasting blood samples was collected as normal venous blood samples.

3.5.4 Preanalytical handling of blood samples

All EDTA tubes were centrifuged at 2200xG for 10 minutes at 4ºC within 15 minutes after collection. The serum tubes were stored at room temperature for 30-60 minutes after collection, before centrifuged at 2200xG for 10 minutes at 20ºC. Further, the samples were distributed into Sarstedt tubes and biobank tubes, and temporarily stored in a freezer at -20ºC. Both serum and plasma were transferred to -80ºC at the end of the day. One serum sample from each time point were temporarily stored in a refrigerator at +4ºC for daily transportation to the laboratory, together with the additional EDTA-blood collected at baseline and at the 24-hour timepoint. The SOP for preanalytical handling of blood samples is described in **appendix 5.**

3.5.5 Assessment of appetite

To assess the experience of hunger, satiety, and desire to eat, all participants were asked to fill out a visual analog scale (VAS) after each blood sampling. The scale used in the present study were based on three straight lines of 100 mm, one for the experience of hunger, one for satiety and one for desire to eat, with words anchored at each end expressing the most positive and the most negative rating/the extremes. All three experiences were graded from "not at all" at the left end of the line to "very" at the right end, and subjects were asked to make a mark across each line corresponding to their feelings. Participants could neither see or refer to their previous ratings when marking the VAS and did not discuss or compare their ratings with each other. The result was read in number of millimeters from 0 to 100, from the left end of the line to the mark. The VAS is shown in **figure 2**.

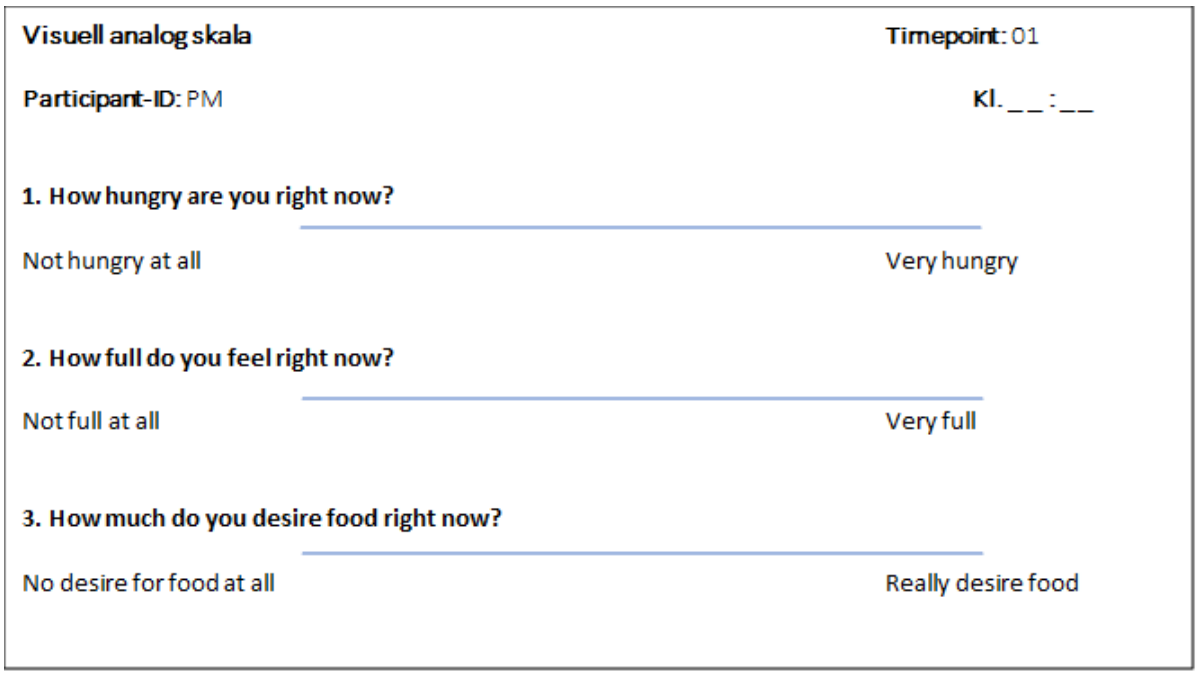


Figure 2 Visual analogue scale used for assessment of perceived hunger, satiety, and desire for food in the PoMet study.

3.5.6 Body composition measurement

Body composition was measured using a BodPod, an Air Displacement Plethymography (ADP) system that determine body composition (fat versus fat-free mass) using whole body densitometric principles (66). The BodPod is considered the gold standard for body composition assessment. The system was calibrated prior to all assessments. Before entering the BodPod, participants were instructed to put on a swimming cap and minimal clothing (e.g., single-layer swimwear without padding, synthetic underwear, compression shorts). Basic subject information was entered into the configured computer system at startup. Further, the participants' bodyweight was measured using the integrated digital scale, followed by a measurement of the body volume while sitting inside the BodPod chamber. While the subject remains in the enclosed space, the system measures the volume of air inside the chamber and calculates body volume indirectly by subtracting this volume from the total air volume when the chamber is empty (66). All participants were instructed to sit quietly while the airflow measurements were conducted. Once the body density was determined, the BodPod predicted the thoracic gas volume (TGV) of the participant and further calculated the percentage of fat and fat-free mass using user-customized densitometric equations. All test results were displayed at the configured computer system and the results was printed. The SOP for measurement of body composition is described in **appendix 6.**

3.6 Biochemical analytes

A targeted metabolomics approach was used to quantify metabolite concentrations of glucose, insulin, and ketones in blood samples from all 14 time points. Capillary blood glucose was assessed by the researchers on site using a handhold HemoCue Hb 201+ Analyzer, whereas concentrations of serum insulin were quantified through immunoassay at the central laboratory of Haukeland University Hospital Bergen, Norway, after completion of the study visits. Acetoacetate and beta-hydroxybutyrate was analyzed by Bevital AS, using gas chromatograph in combination with tandem mass spectrometry (GC-MS/MS). Reference values for reported biomarkers are presented in **Table 3**.

Table 3 Reference levels for biomarkers reported at baseline.

Biomarkers	**Reference levels[3]**	
	Male	**Female**
HbA1c, mmol/mol	≤ 48	≤ 48
Glucose[1], mmol/L	4.0 – 6.0	4.0 – 6.0
Insulin, mIU/L	< 29.1	< 29.1
Betahydroxybutyrate[2], µmol/L	20-700	20-700
Acetoacetate[2], µmol/L	10-200	10-200
HDL, mmol/L	0.8 – 2.1	1.0 – 2.7
LDL, mmol/L	1.8 – 5.7	1.8 – 5.7
Triglycerides, mmol/L	0.45 – 2.60	0.45 – 2.60

HbA1c; Hemoglobin A1c, HDL; High density lipoprotein, LDL; Low density lipoprotein
[1]Reference level in the fasting state
[2]Reported values from Bevital.no
[3]Reference levels retrieved from analyseoversikten.no

3.7 Statistical considerations

All statistics was performed using R version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria, URL: http://www.R-project.org/). In context of/regarding measurements with great variances, where mean values are low and negative values are not possible, skewed distributions with a longer tail towards higher values are common, as is the case for biological measurements (67). Hence, all reported metabolite concentrations were log-transformed prior to analysis and described using the back-transformed geometric mean (gMean) and geometric standard deviation (gSD) as recommended by Limpert and colleagues (67, 68). Anthropometric baseline characteristics were presented as arithmetic means ± SD.

As a measure of uncertainty, the geometric mean metabolite concentrations were accompanied by 95% geometric confidence intervals (gCI), calculated using the geometric standard error (gSE) and formulas 1-3:

$$1)\ \ gSE = gSD^{\frac{1}{\sqrt{n}}}$$

$$2)\ \ 95\%\ CI\ lower\ limit = \frac{gMean}{gSE^{1.96}}$$

$$3)\ \ 95\%\ CI\ upper\ limit = gMean\ x\ gSE^{1.96}$$

The main objective was answered graphically, by plotting metabolite concentrations as a function of time, with the mean time-course indicated by superimposing the geometric mean concentrations (95% gCI) on top of the individual data. Scatterplots with smoothed LOESS curve (locally estimated scatterplot smoothing) were made with the geom_smooth() function from the ggplot-2 package, that fits LOESS curve to the data, and spearman correlation (ranked correlation) was calculated between variables.

3.8 Ethics

3.8.1 Ethics and safety

The study was conducted in accordance with the Helsinki-II declaration and has been approved by The Regional Committee for Health Research Ethics (REK 236654). Prior to the study visit, participants received the consent form by email to allow sufficient time to read and understand the protocol. At attendance, the project staff orally went through the risk and burdens of participating in the study with the participants and explained that they at any time were free to withdraw from the study with no obligation to state a cause. Before signing the consent form, participants were explicitly given the opportunity to ask additional questions. Participants that communicated a great discomfort during the study visit, either in oral or by body language (for instance syncope associated with blood sampling or fasting), were eliminated from the study and immediately offered food and drinks. Blood test results from the baseline sample and the 24 h sample was evaluated short time after the study, and participants with abnormal blood test results were contacted.

3.8.2 Quality assurance

The study was carried out at the Research Unit for Health Surveys, a core facility at the University of Bergen, Norway, and all study procedures were done according to Good

Clinical Practice. Blood samples was conducted by qualified personnel, and all clinical-chemical analyses was performed at the central laboratory of Haukeland University Hospital Bergen, Norway (certified laboratory NSEN-ISO 15189), and by Bevital AS.

4. Results

4.1 Recruitment

A flowchart of the recruitment process is presented in **Figure 3**. 36 healthy, young males (n=18) and females (n=18) were recruited in the study. Three female participants dropped out during the study visit, whereof two at baseline, and one withdrew two hours into the study visit, due to difficulties with blood sampling. Hence, 33 participants completed the study (18 male, 15 female). Data from the participant withdrawing after two hours is included in the analyses, resulting in a total sample size of 34. Hence, descriptive baseline characteristics represents data on 34 participants, whereas the data analysis consists of 33 subjects.

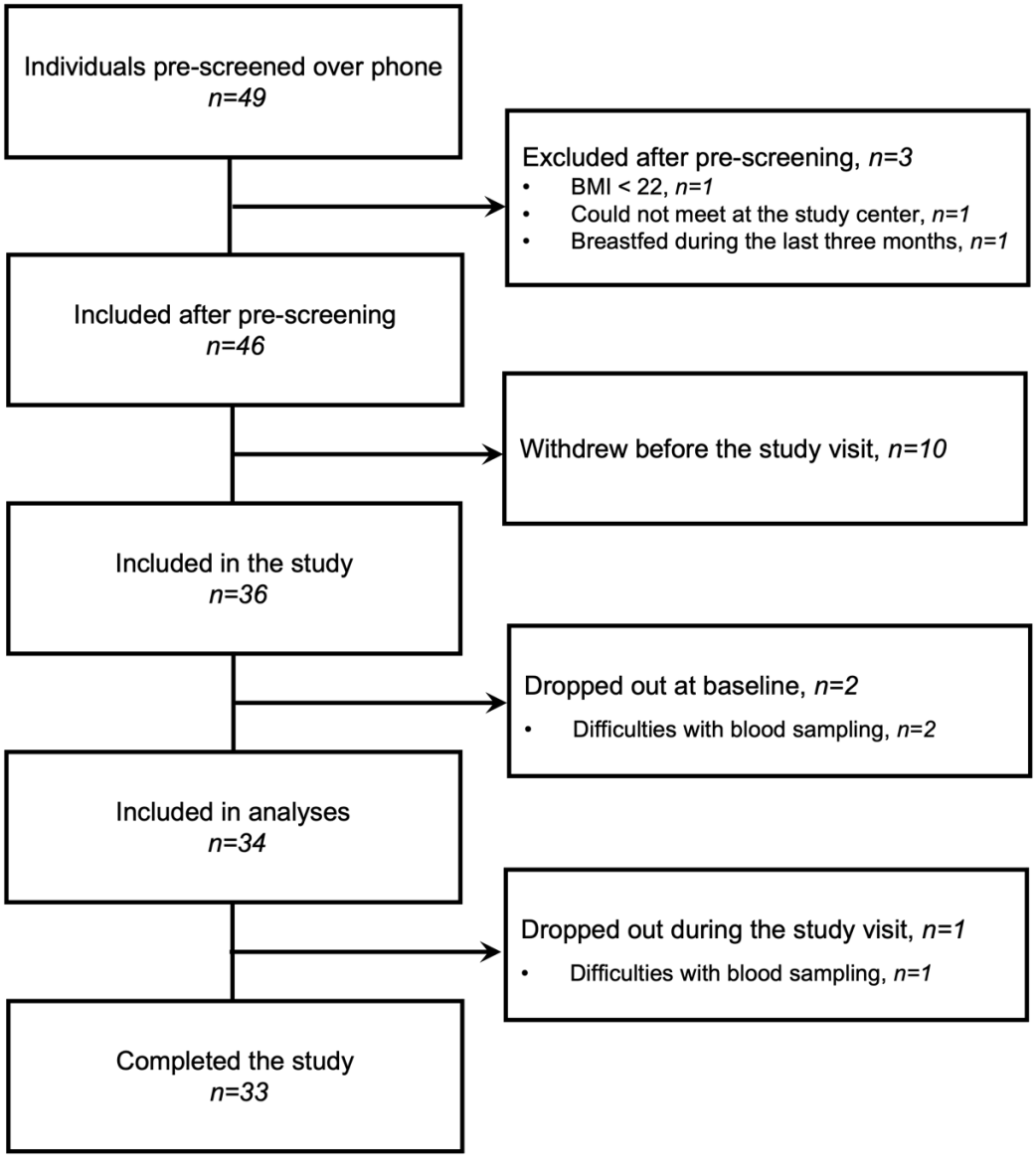


Figure 3 Flowchart of the recruitment process in the PoMet study.

4.2 Participant characteristics

Descriptive baseline characteristics for males, females, and the total study population, including anthropometric measurements and relevant biomarkers, are summarized in **Table 4**. As expected, there were difference in body composition between genders. Height, body mass and waist circumference were slightly higher among the male participants, whereas fat mass percentage was in favor of the females. Consequently, males had grater resting metabolic rate (RMR) compared to female participants. However, BMI was similar between males and females. Moreover, on an average, the male participants were two years older than the female participants. Furthermore, except from one female HDL-concentration (2.8 mmol/L) and three blood glucose-values (6.1, 6.2 and 6.4 mmol/L) slightly above the reference range, and three LDL-values (1.6, 1.7 and 1.7 mmol/L) slightly below the reference range, routine clinical measurements for all participants were within the reference range at baseline.

Table 4 Baseline characteristics for males, females, and the total study population in the PoMet study.

Characteristics	Mean (SD)			Percentiles (total, n = 34)			Extremes (total, n = 34)	
	Male (n = 18)	Female (n = 16)	Total (n = 34)	25%	50%	75%	Minimum	Maximum
Age, year	26.2 (2.8)	24.6(3.0)	25.5 (3.0)	22.9	25.8	28.0	20.0	30.4
Height, cm	184.4 (5.5)	168.0 (7.6)	176.6 (10.5)	169.2	178.3	184.8	152.5	194.7
Body mass[1,2], kg	81.3 (8.0)	65.5 (6.0)	74.1 (10.7)	63.4	74.8	80.0	57.2	98.3
BMI, kg/m^2	23.9 (1.7)	23.1 (1.5)	23.5 (1.6)	22.0	23.5	24.8	20.2	26.9
Waist circumference, cm	88.3 (6.8)	74.7 (4.3)	81.9 (8.9)	76.6	80.5	88.9	67.3	100
Fat mass[2], %	19.4 (7.4)	29.0 (5.3)	23.8 (8.1)	15.7	24.9	30.0	10.5	41.9
RMR[2], kcal	1747 (163)	1277 (145)	1533 (283)	1270	1552	1741	1053	2200
Biomarkers	**gMean (gSD)**			**Percentiles (total, n = 34)**			**Extremes (total, n = 34)**	
	Male (n = 18)	**Female (n = 16)**	**Total (n = 34)**	**25%**	**50%**	**75%**	**Minimum**	**Maximum**
HbA1c, mmol/mol	31.8 (1.10)	30.7 (1.11)	31.3 (1.11)	29.3	32.0	34.0	24	39
Glucose, mmol/L	5.40 (1.09)	5.12 (1.10)	5.27 (1.10)	4.90	5.20	5.68	4.5	6.4
Insulin, mIU/L	4.53 (1.76)	4.37 (2.17)	4.45 (1.94)	3.40	4.40	7.87	1.0	11.2
Betahydroxybutyrate, µmol/L	63.8 (2.37)	61.2 (1.89)	62.5 (2.12)	34.0	68.8	98.9	17.3	398.5
Acetoacetate, µmol/L	40.9 (2.08)	33.8 (1.65)	37.4 (1.88)	22.9	36.6	50.8	13.1	166.8
HDL, mmol/L	1.45 (1.20)	1.66 (1.26)	1.55 (1.24)	1.30	1.50	1.78	1.0	2.8
LDL, mmol/L	2.58 (1.39)	2.44 (1.21)	2.51 (1.31)	2.00	2.50	3.18	1.6	4.3
Triglycerides, mmol/L	0.90 (1.46)	0.81 (1.46)	0.85 (1.46)	0.63	0.81	1.17	0.50	1.82

Abbreviations: BMI; Body mass index, RMR; Resting metabolic rate, HbA1c; Hemoglobin A1c, HDL; High-density lipoprotein, LDL; Low-density lipoprotein.
[1]Although changes in body mass was tracked using a Seca scale at three time points during the intervention, the body weight measure from the body composition analysis was used for participant characteristics as the BodPod-scale (was calibrated prior to each analysis and) is considered the most accurate scale.
[2]Estimated using Bod Pod

4.3 Postprandial biomarker concentration

Figure 4-7 presents quantified serum concentrations of glucose, insulin, acetoacetate, and betahydroxybutyrate, individually plotted as a function of time since completion of the standardized breakfast meal.

4.3.1 Blood glucose

Blood glucose concentrations after completion of the standardized breakfast meal are graphically displayed as a function of time in **Figure 4**. As expected, the largest fluctuations were observed during the first two hours postprandially, with gMean (gSD) glucose concentration peaking 15 minutes after food consumption (7.47 [1.10] mmol/L). Three hours after completion of the standardized breakfast, the gMean concentration stabilized at levels similar to baseline levels, followed by a modest, but steady decline throughout the intervention. At 24 hours, the gMean concentration was slightly lower than for the baseline measurement, with a difference of 0.72 mmol/L.

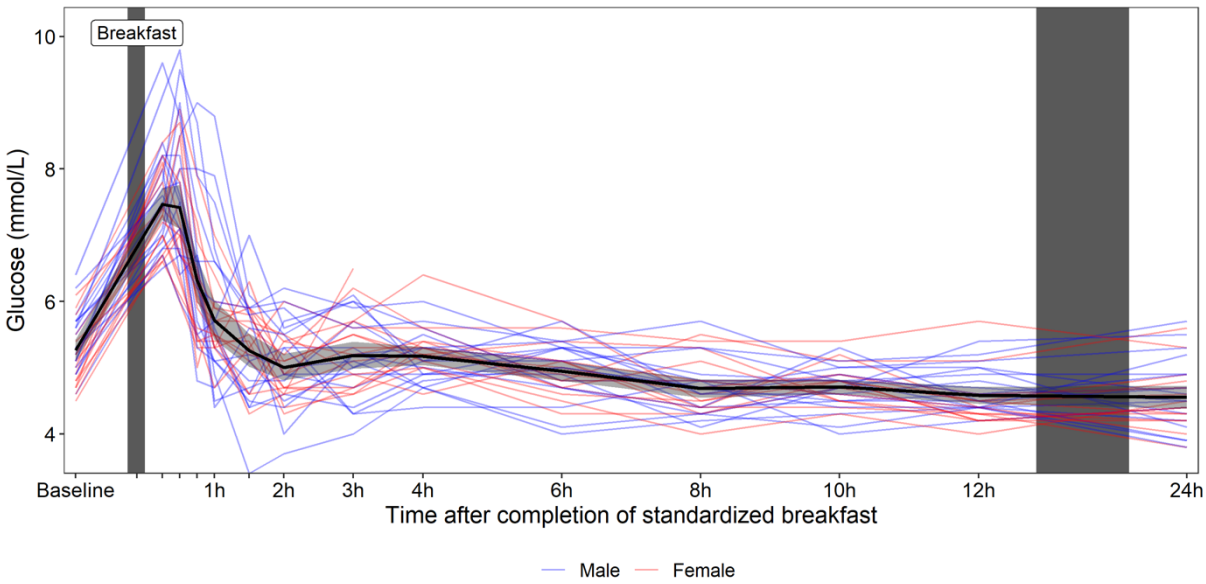


Figure 4 Serum glucose concentrations (mmol/L) as a function of time since completion of the standardized breakfast meal. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.3.2 Insulin

Figure 5 graphically presents the measured concentrations of serum insulin as a function of time since completion of the standardized breakfast meal. As expected, and in accordance with the changes in circulating glucose concentrations, the biggest insulin fluctuations were observed within two hours after the meal. Overall, insulin concentrations increased immediately after food consumption, peaking 30 minutes postprandially (41.3 [1.59] mIU/L), before a distinct decrease towards baseline values for the five subsequent measurements. After four hours of fasting, insulin concentration dropped below the baseline value (3.81 [2.45] mIU/L), followed by a modest, but steady decline throughout the intervention. At 24 hours, circulating insulin concentration was 2.00 [2.09] mIU/L.

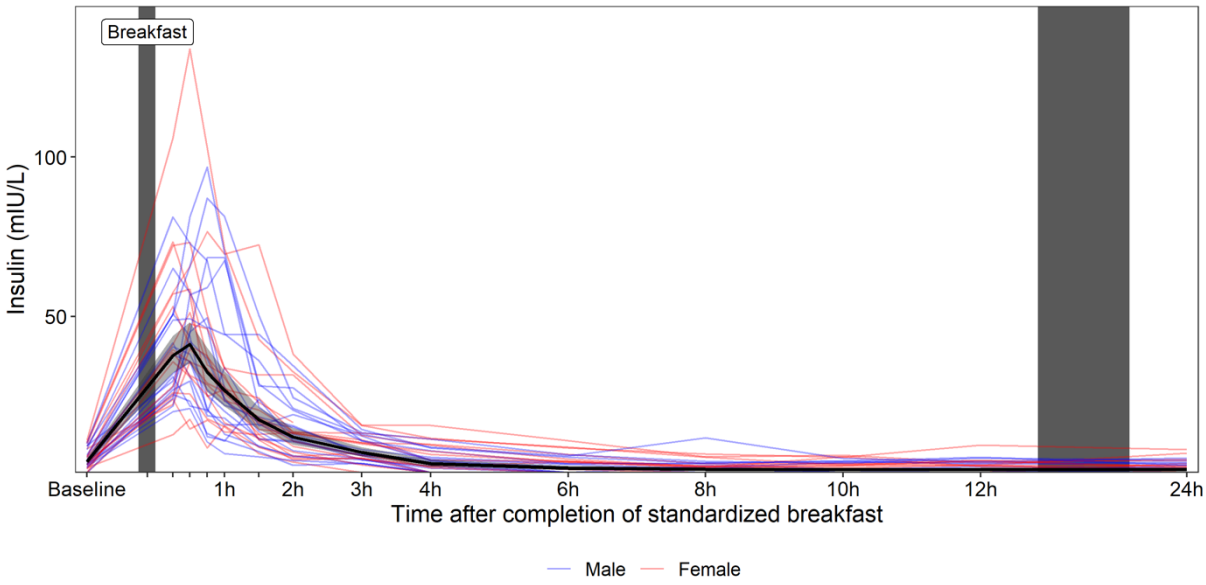


Figure 5 Serum insulin concentrations (mIU/L) as a function of time since completion of the standardized breakfast meal. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.3.3 Ketones

4.3.3.1 Acetoacetate

Figure 6 graphically illustrated the quantified concentration of acetoacetate plotted as a function of time after completion of the standardized breakfast meal. Overall, there was a steady increase in circulating acetoacetate concentrations throughout the intervention. From baseline until 24 hours after meal consumption, gMean (gSD) concentrations increased from

37.4 [1.88] µmol/L to 204.0 [1.95] µmol/L, corresponding to a difference of 166.6 µmol/L. Interindividual differences increase in line with the number of hours from meal consumption. 24 hours postprandially, minimal and maximal circulating concentrations was 20.9 µmol/L and 678.4 µmol/L, respectively.

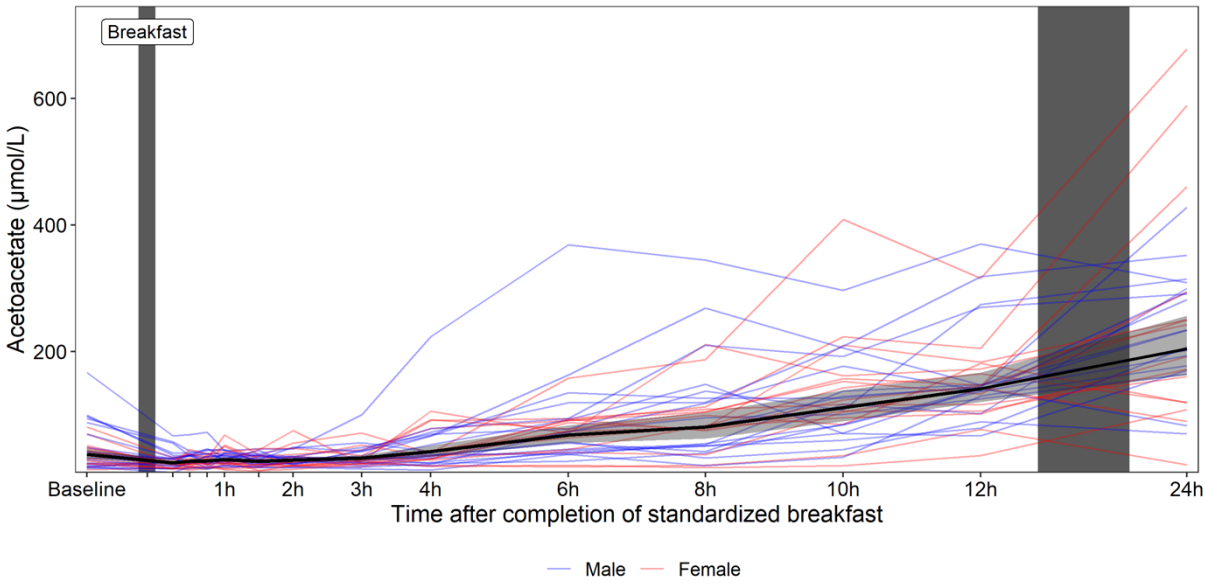


Figure 6 Serum acetoacetate concentrations (µmol/L) as a function of time since completion of the standardized breakfast meal. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.3.3.2 Betahydroxybutyrate

Quantified concentrations of betahydroxybutyrate as a function of time since completion of the standardized breakfast meal are displayed in **Figure 7**. Baseline concentrations range from 17.3 µmol/L to 398.5 µmol/L. Overall, there was a slight decrease in circulating betahydroxybutyrate concentrations for the first six measurements, with the lowest gMean (gSD) concentration (23.09 [1.27] µmol/L) measured 90 minutes post food consumption. From this point, serum betahydroxybutyrate concentrations followed a steady increase throughout the intervention, with a peak gMean value at 24 hours (463.54 [2.11] µmol/L). Interindividual differences increased in line with the number of hours from meal consumption, with the greatest variation at 24 hours after completion of the meal. The minimal and maximal circulating concentrations at 24 hours, was 49.3 µmol/L and 1533.3 µmol/L, respectively.

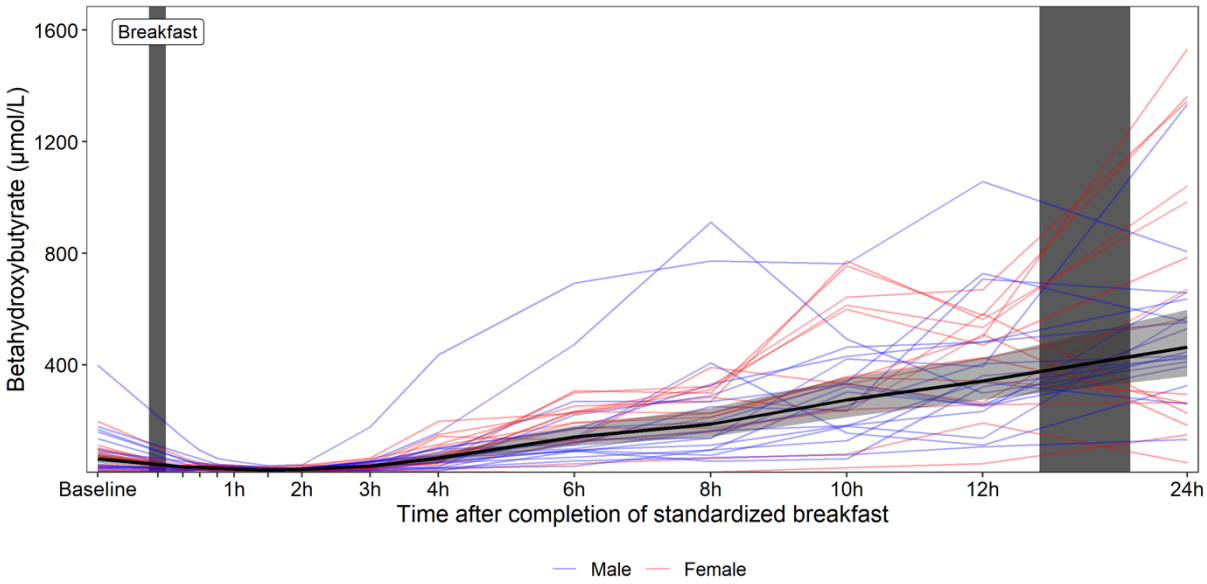


Figure 7 Serum betahydroxybutyrate concentrations (µmol/L) as a function of time since completion of the standardized breakfast meal. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.4 Time resolving presentation of perceived hunger, satiety, and desire for food

VAS results on perceived hunger, satiety and desire for food are individually plotted as a function of time since completion of the standardized breakfast meal in **Figure 8-10**.

4.4.1 Hunger

Figure 8 shows VAS results on perceived hunger as a function of time since completion of the standardized breakfast meal. The figure shows great interindividual variation throughout the intervention, clearly expressed through the broad 95% geometric confidence interval. Overall, hunger follows a gradual increase from 45 minutes postprandially, peaking at 8 hours post meal consumption. After 8 hours, the feeling of hunger decreased until 10 hours postprandially, before reaching a plateau throughout the remaining measures.

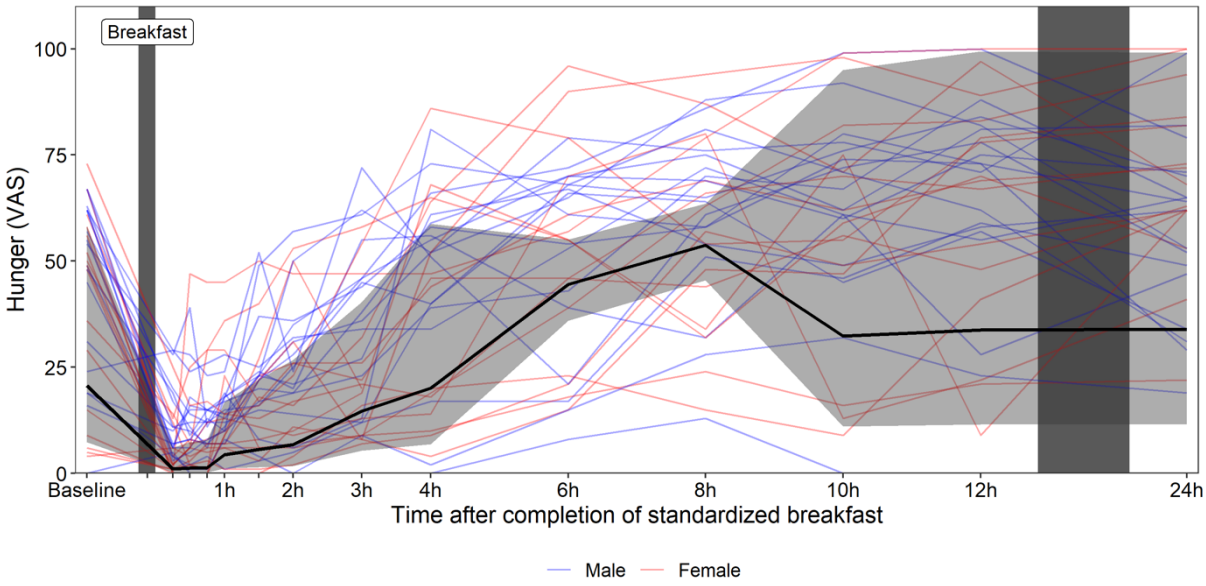


Figure 8 Perceived hunger as a function of time since completion of the standardized breakfast meal, assessed using VAS. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.4.2 Satiety

VAS results on perceived satiety as a function of time after completion of the standardized breakfast meal are displayed in **Figure 9**. As for hunger, data on satiety shows great interindividual variations. Overall, as expected, the highest satiety feeling was reported 15 minutes post meal consumption. Following the mean, a remarkably drop in satiety was observed one hour after completion of the standardized breakfast, followed by a more gradual decrease throughout the remaining measurements. From eight hours, the mean value was close to zero, reflecting an absence of perceived satiety in majority of participants.

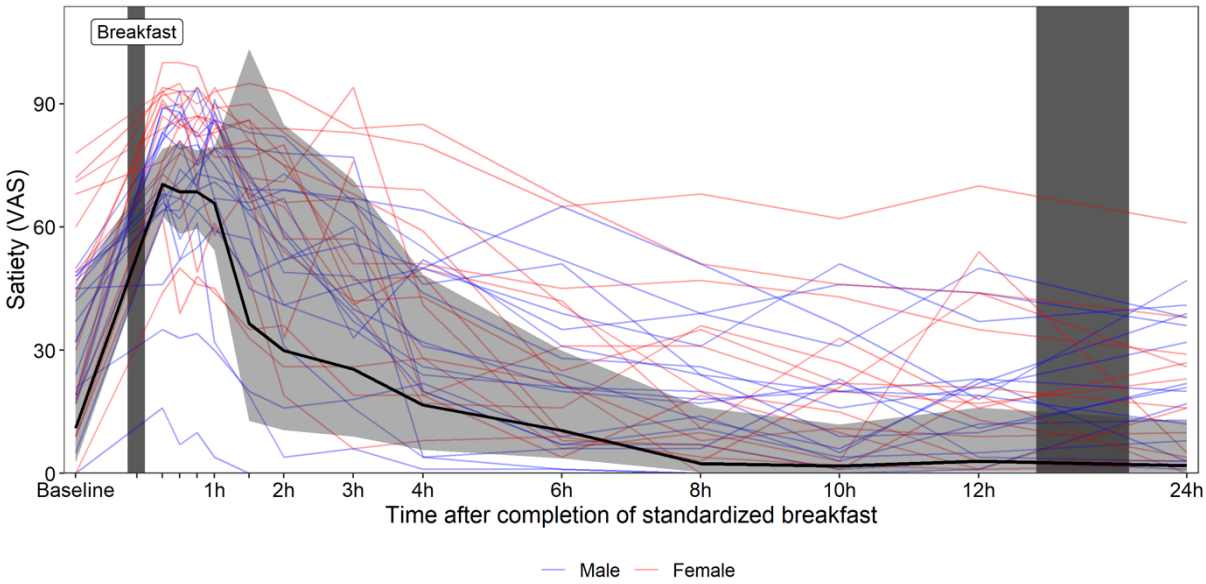


Figure 9 Perceived satiety as a function of time since completion of the standardized breakfast meal, assessed using VAS. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.4.3 Desire for food

Figure 10 presents VAS results on desire for food as a function of time after completion of the standardized breakfast meal. Coinciding with the reported results on hunger and satiety, the overall desire for food is modest during the first 45 minutes post food consumption. After 45 minutes, the feeling of desire progressively increased for the next 8 measurements, before reaching a plateau at 10 hours, which persisted throughout the intervention. As for hunger and satiety, desire for food shows great inter- and intraindividual differences.

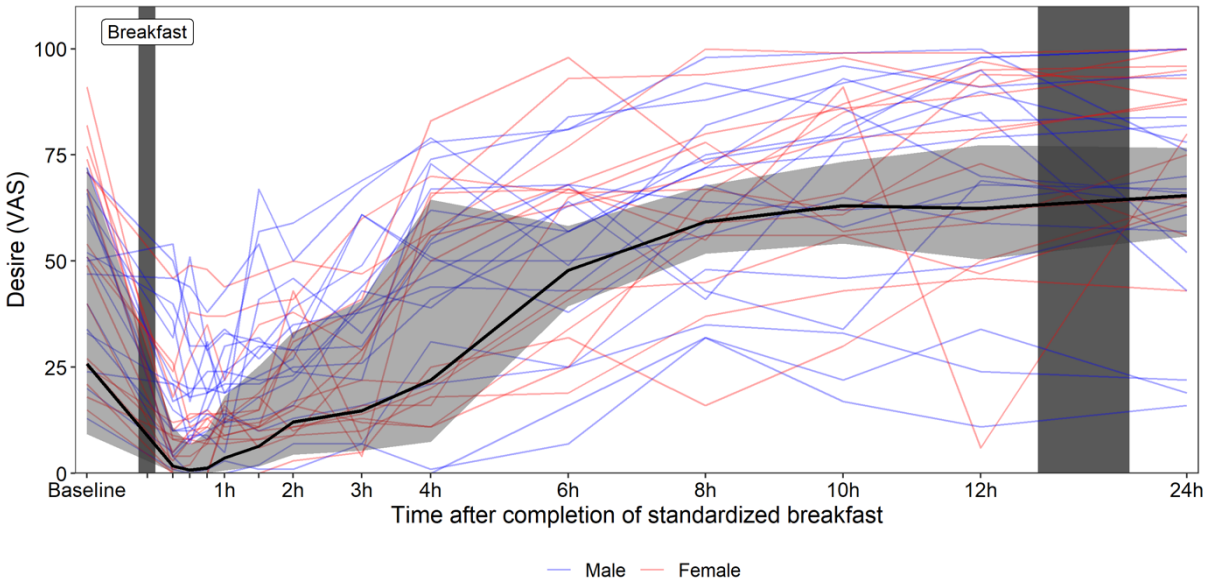


Figure 10 Desire for food as a function of time since completion of the standardized breakfast meal, assessed using VAS. The black line represents the geometric mean concentration for the total study population. Male participants are represented as blue lines, whereas female participants are represented as red lines. The shaded, grey area around the black line illustrates the 95% geometric confidence interval. The vertical line to the left indicates the time of meal consumption and the initial time of intervention. The broader vertical line to the right indicates time spent outside the study center.

4.5 Correlations between glucose and perceived appetite

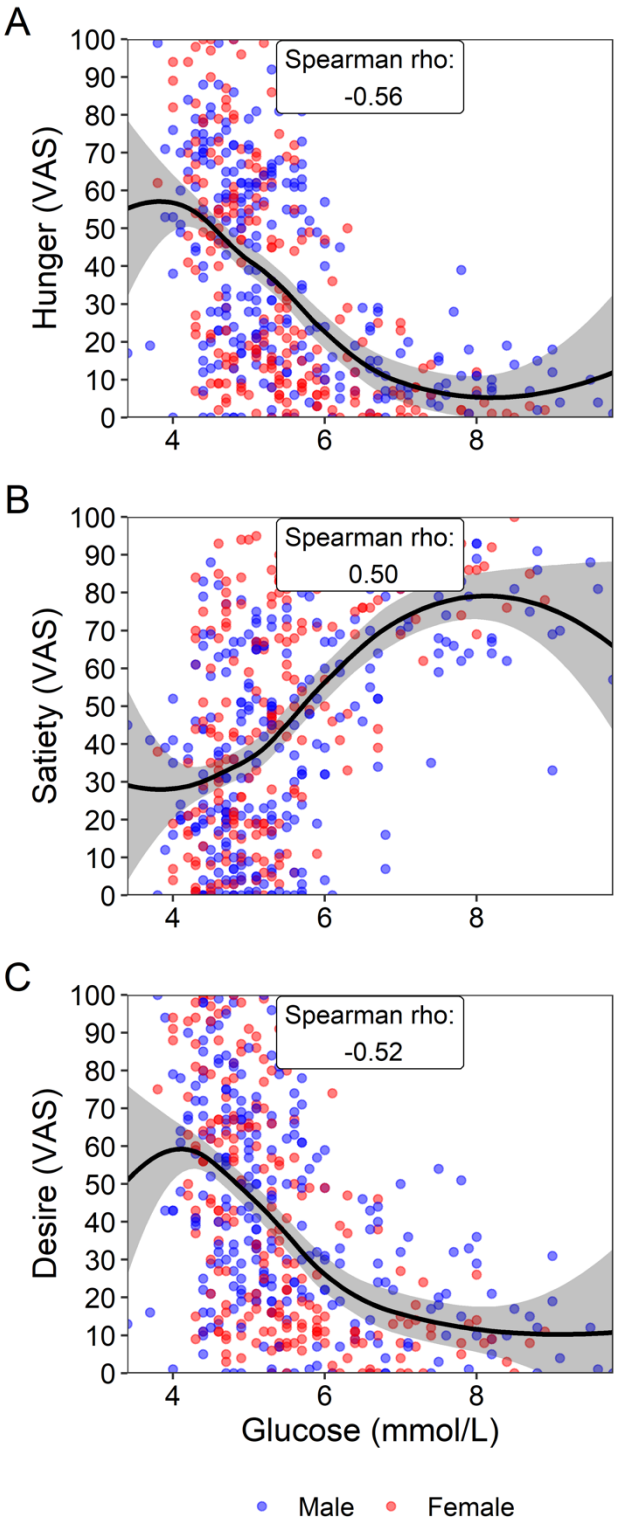
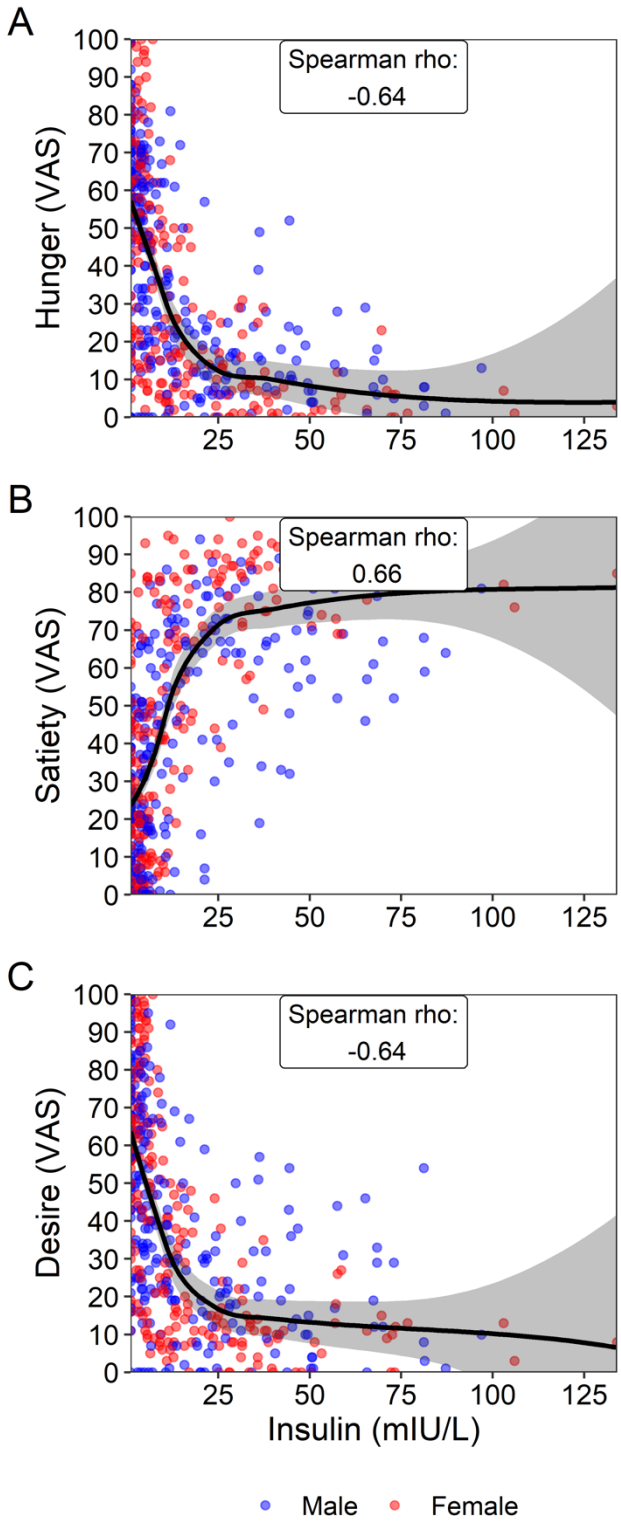


Figure 11A-C respectively represents the observed relationships between measured blood glucose concentrations and VAS results on hunger, satiety, and desire for food. All three scatterplots show a relatively linear relationship in the area where most data are collected, corresponding to glucose concentrations between 4 and 6 mmol/L. The correlation coefficient between hunger and circulating blood glucose gives a strong negative correlation between the two variables (spearman rho: -0.56), indicating that higher concentrations of glucose is associated with lower reported hunger among participants. In contrast to the negative correlation between hunger and circulating blood glucose, the correlation coefficient between satiety and blood glucose indicates a positive correlation (spearman rho: 0.50). In this case, higher circulating glucose concentrations correspond to higher reported satiety. Finally, regarding desire for food, the correlation coefficient gives a strong negative correlation with circulation blood glucose (spearman rho: -0.52), implying that higher concentrations of glucose correspond to lower desire for food among participants.

Figure 11 Scatterplots between glucose and hunger (A), satiety (B), and desire for food (C). Male participants are presented as blue points, whereas females are represented as red points. The black lines represent a smoothed LOESS curve through the data. The shaded, grey area around the black line illustrates the 95% confidence interval. Spearman correlation is calculated and presented in the plots.

4.6 Correlations between insulin and perceived appetite



The scatterplots in **Figure 14-16** shows the relationship between measured serum concentrations of insulin and VAS results on hunger, satiety and desire for food, respectively. All curves show a strong correlation for insulin concentrations up to 25 mIU/L, before they tend to flatten into more horizontal lines. Regarding hunger, the correlation coefficient gives a negative correlation with circulating insulin concentrations (spearman rho: -0.64), indicating that higher concentrations of insulin correspond to lower reported feelings of hunger. Contrarily, the strongly positive spearman correlation between satiety and serum insulin concentrations gives a positive relationship (spearman rho: 0.66), implying that high concentrations of insulin tend to coincide with high ranks of satiety. Lastly, as for hunger, the correlation coefficient between desire for food and measured serum concentrations of insulin gives a strong negative correlation (spearman rho: -0.64), indicating that higher concentrations of insulin correspond to lower reported desire for food among participants.

Figure 12 Scatterplots between insulin and hunger (A), satiety (B), and desire for food (C). Male participants are presented as blue points, whereas females are represented as red points. The black lines represent a smoothed LOESS curve through the data. The shaded, grey area around the black line illustrates the 95% confidence interval. Spearman correlation is calculated and presented in the plot.

4.7 Correlations between ketones and perceived appetite

Figure 13 display six different scatterplots on the correlations between circulating ketones and reported parameters of appetite. The nature of all the curves shows a strong correlation for concentrations up to 100 μmol/L, before they tend to flatten into more horizontal lines. **Figure 13A** and **Figure 13B** illustrates the observed relationship between VAS results on hunger and measured serum concentrations of betahydroxybutyrate and acetoacetate, respectively. In context of hunger, the correlation coeffisients indicate a strong positiv correlation for both metabolites (spearman rho: 0.58 for betahydroxybutyrate and 0.55 for acetoacetate), giving that higher circulating concentrations correspond to higher reported hunger. Further, **Figure 13C** and **Figure 13D** respectively presents the relationship between VAS results on satiety and quantified serum concentrations of betahydroxybutyrate and acetoacetate. In contrast to the positive correlation with hunger, the downward slope on the scatterplots between satiety and both metabolites conform to strong negative correlations (spearman rho: -0.55 for betahydroxybutyrate and -0.48 for acetoacetate), indicating that higher circulating concentrations correspond to lower feelings of satiety. Finally, **Figure 13E** and **Figure 13F** illustrates the observed relationship between VAS results on desire for food and measured serum concentrations of betahydroxybutyrate and acetoacetate, respectively. As for hunger, the relationship between desire for food and serum concentrations of both metabolites gives a strong positive correlation (spearman rho: 0.61 for betahydroxybutyrate and 0.57 for acetoacetate). The upward slope on the scatterplots indicate that higher circulating concentrations tend to coincide with higher desire for food among participants.

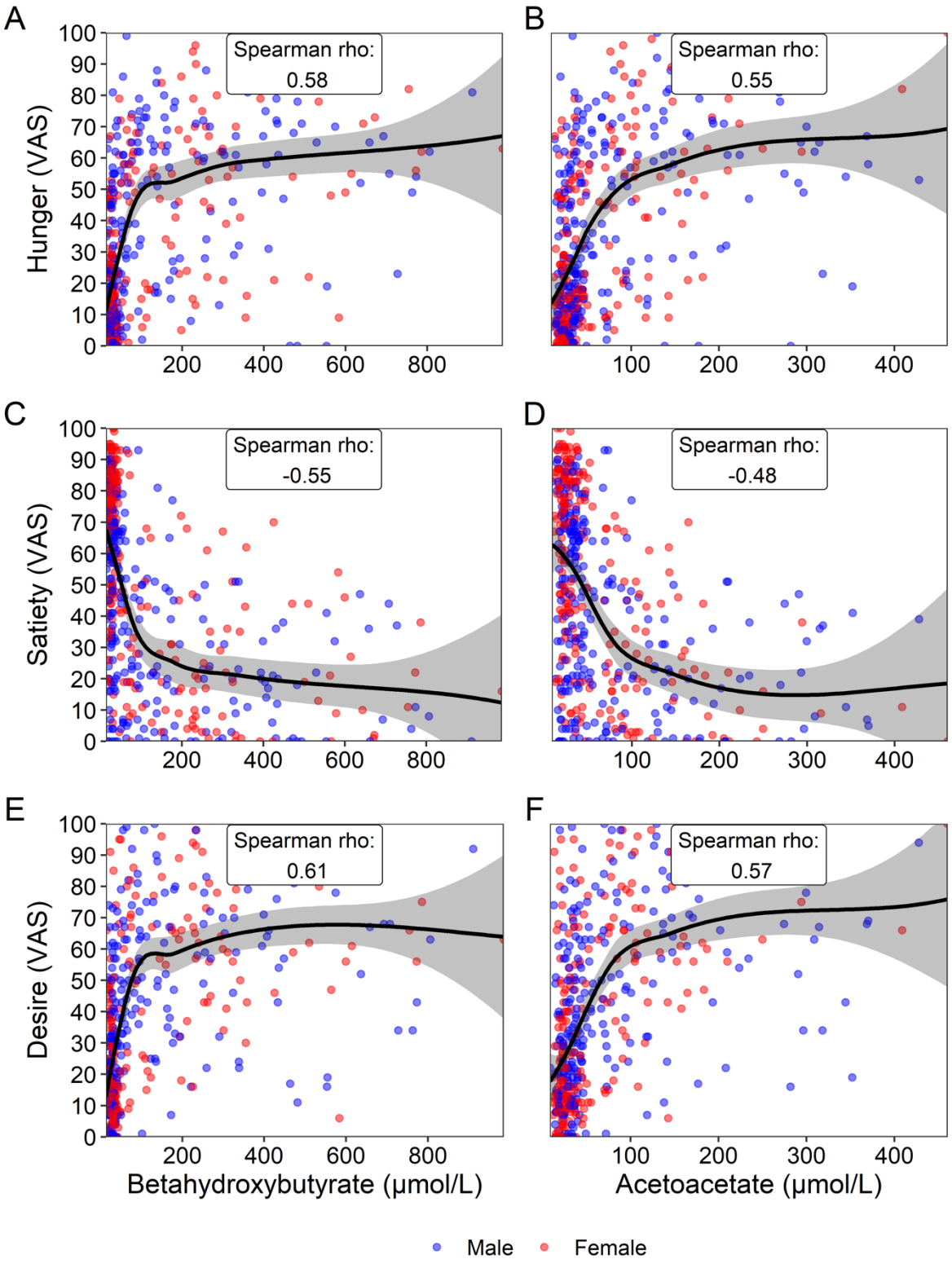


Figure 13 Scatterplots between betahydroxybutyrate and hunger (A), satiety (C), and desire for food (E) are displayed in the left column, and scatterplots between acetoacetate and hunger (B), satiety (D), and desire for food (F) are displayed in the right column. Male participants are presented as blue points, whereas female participants are represented as red points. The black lines represent a smoothed LOESS curve through the data. The shaded, grey areas around the black lines illustrates the 95% confidence interval. Spearman correlation is calculated and presented in the plots.

5. Discussion

The main objective of the present thesis was to investigate how concentrations of blood glucose, insulin, and ketones fluctuates during the postprandial period, and to explore how perceived hunger and satiety changes during postprandial adaptation, and how this relates to circulating concentrations of the above-mentioned metabolites. The thesis aimed to look closer into the mechanisms of appetite regulation during a longer period of food deprivation, focusing of the potential impact of the metabolic switch and the onset of ketone body production in the postprandial period.

5.1 Summary of main findings

Overall, we observed an expected increase in concentrations of glucose and insulin immediately after food consumption, peaking at approximately one hour, before returning to baseline values. For the ketones, the lowest concentrations were observed during the early postprandial period, followed by a gradual increased throughout the intervention. Moderate to strong correlations were observed between the biomarkers and the parameters of perceived hunger, satiety, and desire for food. In general, glucose and insulin were associated with less hunger and more satiety, while the opposite was observed for the ketones.

5.2 Methodological aspects and considerations

As for all clinical research, some strengths and limitations should be considered when interpreting the results.

5.2.1 Study design

A strength to the current study is the nature of the study design. As opposed to an observational study where the subjects are recruited for blood sampling after consumption of different meals at different times of the day, and hence with a different number of hours since completion of the last meal, an intervention study makes it possible to standardize food intake, meal composition, and timing of the meal. An additional advantage of the study design is the non-existing problem with loss to follow-up, as the participants only were asked to attend one single study visit. Furthermore, with one baseline measure and 13 repeated measurements throughout the postprandial period of 24 hours, the results of the current study covers the entire period of interest.

Nonetheless, the nature of the study makes it difficult to know whether the measured concentrations and the associated metabolic adaptations gives a representative reflection of the everyday metabolic situation for each subject. In addition to the observed variations in biomarker concentrations and perceived hunger, satiety, and desire for food throughout the day, the parameters under study are highly sensitive to day-to-day variations. Multiple factors, including fluid intake, dietary intake, food choices, state of disease, use of medications, and amount of physical and everyday activity, are likely to somehow influence the measurements in either direction. The potential impact of all the above-mentioned aspects, suggests a need for longer study duration with repetitive interventions in future research, in order to account for day-to-day variations and strengthen the validity of the results. However, more than one study visit for each subject would require far more of the participants and are likely to result in a more challenging recruitment process.

5.2.2 Preparations in advance of attendance

To standardize physiological and metabolic conditions at attendance, and to maximize between-subject comparability, participants were asked to abstain from alcohol and avoid strenuous physical activity for the last 24 hours before study visit, which can be seen as a strength to the study. Additionally, participants were instructed to not use any dietary supplements the week before attendance, and to eat an evening meal analogous to the standardized breakfast meal at 8 PM the prior evening, as recommended by LaBarre et al. (69). Participants reported on varying degree of compliance to the given instructions, especially regarding food choice and composition of the standardized evening meal, which is a potential cause for greater interindividual difference in the metabolome and hence threatens the between-subject comparability. However, as the participants attended the study center in an approximate 12 hour overnight fasting state, the composition of the evening meal should not be the decisive factor for the postprandial adaptation throughout the intervention after completion of the standardized breakfast meal. Nonetheless, to provide an opportunity to compare 12 hours of night fasting with 12 hours of day fasting and thus be able to interpret the possible effects of circadian rhythms, a thorough standardization of the last evening meal is of great importance.

5.2.3 The standardized breakfast intervention

Another phenomenon under consideration is the large interindividual variation in postprandial metabolic response. In the present study, the standardized breakfast meal was composed to harmonize with an average Norwegian diet regarding food choice and composition of macronutrients. All meals were prepared by the same project staff in accordance with a SOP, and the participants were instructed to use the same amount of time to complete the meal. However, despite of identical food intake and meal setting, large interindividual variability in postprandial metabolic responses of blood glucose and insulin concentrations is a challenge (70). Berry and colleagues reported that person-specific factors, including gut microbiome and genetic variants, had a greater influence than did meal macronutrients on postprandial adaptation (70). Furthermore, our results cannot be extrapolated directly to other meals with different food compositions, nor to meals consumed at different times of the day, as the meal composition and the potential impact of circadian rhythm is of relevance to the given response.

The breakfast meal was supposed to cover approximately 24% and 20% of daily energy needs, for female and male participants, respectively. However, based on estimated RMR from the BodPod measurements, the results showed great variation in regards of energy needs. Hence, the standardized breakfast made up a various percentage of the participants' daily energy requirements. Furthermore, a considerable difference in body composition was observed between subjects. Differences in the ratio of fat mass to fat-free mass may affect how fast a meal is metabolized, and further impact the circulating concentrations of glucose and insulin. Ideally, all participants should have received a breakfast meal corresponding to their BMI and estimated BMR/RMR, as has been done previously by several studies, but for practical reasons we chose to standardize the intervention meal, which can be discussed as a weakness. However, the use of identical meals as a standardized intervention correspond to equal consumption of the different nutrients, which makes it easier to compare the postprandial adaptations.

5.2.4 Data collection

5.2.4.1 Assessment of perceived appetite

A strength to the use of VAS in the present study is the possibility to assess the degree of a subjective feeling. Compared to alternative approaches like the widely used Likert scales,

typically involving 3-7 responses with different degrees of agreement and disagreement, the continuous nature of the VAS-scale makes it possible to give a more thorough differentiation of the perceived sensations. The refined level-gradations makes the VAS-method more sensitive and more accurate for detecting small changes (71). Furthermore, all answers to the evaluative questions were given with no opportunity to discuss the answers with other participants, nor compare the marks with own previous ratings. Thus, all answers were solely based on the perceived feeling of each subject in the current moment, which strengthens the credibility of the ratings.

However, some challenges with the use of VAS needs to be considered. First, the assessment method gives room for large individual variations. In the current study, participants were asked to answer the evaluative questions at every time point for blood sampling, with no further instructions on how to evaluate or grade their perceptions. Hence, all answers were based on subjective definitions of the different sensational concepts, giving room for misinterpretations and great individual variance as the perception of appetite, hunger and satiety integrates a myriad of modalities. In a focus group study by Murray and colleagues, sensations of hunger and satiety were described by the subjects as having both physical and psychological components (72). Overall, hunger was defined both as loss of energy, focus on eating and desire to eat, and in a more physical manner as the presence of hunger pains, stomach growls, or a feeling of emptiness. Contrastingly, satiety was described simply as a physical feeling of stomach stretch or food in the stomach, as lack of desire to eat, and in a more psychological perspective as contentment, satisfaction, or a feeling of being focused and having surplus of energy (72). This suggest that some individuals understand sensations of hunger and satiety simply as polar opposites and emphasize a common misinterpretation of satiety claims as absence of hunger or lack of desire to eat. Hence, it seems that both hunger and satiety is experienced differently between individuals, making within-subject comparisons more accurate and reliable than between-subject comparisons. Furthermore, participants may have different perceptions of the scale. What is perceived as maximum hunger for one participant may be perceived as mediocre by another, which brings further challenge when comparing perceptions between subjects. Second, there was great variance in how much effort the participants put into the given task. Some respondents filled out the forms more diligent and used a lot more time to complete the task when compared to others. Third, the subjects were asked to think through how hungry they were feeling at every time point before answering the evaluative questions. This deviates from a real-life situation and

may, in context of a 24-hour fasting period, influence the answers in favor of feeling hungrier. Fourth, the validity of the results on appetite is difficult to determine, since we do not have an objective measure of hunger, satiety, and desire for food which can be compared to the subjective VAS scores. Lastly, previous studies report that respondents seem to experience difficulties when filling out VAS, having problems understanding how to respond in a continuous line with no divisions (73). Consequently, subjects tend to grade their responses closer to the edge of the line, which may favor responses at odds with what they really were feeling. Alternatively, the tendency for people to respond closer to the extremes, could be a result of modified answers according to more socially desirable responses. Our results suggest some degree of underreporting in regards of hunger, which may be due to a desire to appear unaffected by the situation. However, on the other hand, others may be influenced by the fact that they are not allowed to eat for 24 hours and therefore exaggerate their perceived feeling of hunger.

5.2.4.2 Analytic variation

As for all clinical research, measurement errors may be of concern when analyzing the metabolites of interest. Measurement error refers to the difference between a measured quantity and its true value and includes both systematic and random errors, in which systematic errors are caused by any factors that systematically affect measurement of the variable across the sample and typically arises from an innate flaw in the data sampling technique or measurement instrument, whereas random errors are caused by any factors that randomly affect measurement of the variable across the sample (74). Systematic errors tend to be consistently either positive or negative, and hence distorts the research study findings in a specific direction. Unlike systematic error, random errors do not have any consistent effects across the entire sample. Instead, it fluctuates unpredictably around the true value in both directions, such that some recorded values will be higher than the true values while others will be lower, and are expected to average out to zero when combined (75).The important property of random error is that it adds variability to the data but does not affect average performance for the group. Consequently, random measurement errors lead to reduced precision, indicated by wider confidence intervals around the estimated metabolite concentrations. However, both the central laboratory of Haukeland University Hospital, Bergen, Norway and Bevital AS has reported great within- and between-day reproducibility, which limits the potential uncertainty related to analytic variation in the present study.

5.2.5 Bias

The characteristic of a clinical study to produce valid results, commonly referred to as internal validity, can be affected by random and systematic (bias) errors. Bias is an unavoidable problem in clinical research and can be defined as any deviation leading to incorrect conclusions, resulting from recruitment methods affecting the study participation (selection bias) or systematic distortions during data collection (information bias) (76).

5.2.5.1 Selection bias

Volunteer bias, or self-selection bias, is an aspect of selection bias which occur when subjects who volunteer for a study differ in relevant characteristics from those who do not volunteer (76). As recommended by Ganguli et al., health-related studies with populations composed partly or entirely of volunteers should take a potential volunteer bias into account when analyzing and interpreting data (77). In the present study, the intention was to recruit young, healthy adults from various districts and social groups in Bergen. However, due to practical reasons, the recruitment process was more aggressive in the areas around Haukeland University Hospital. In our experience, this population tends to volunteer for participation in clinical studies more often when compared to others and appears to be highly motivated to contribute to clinical research. This leaves a possibility that the included subjects are more health conscious than the average population, commonly referred to as the healthy volunteer effect (78). Hence, it is not possible to exclude a potential effect of selection bias in the present thesis, which could limit the external validity. Furthermore, the criteria for inclusion and exclusion in the study were relatively narrow, resulting in a homogenous study population comprising overall healthy, young, normal-weight participants. This increases the internal validity but may limit the generalizability of the findings. However, the aim was never to recruit a representative sample, but to recruit the most homogeneous sample possible in order to limit noise due to other variations. With no goal of extrapolating the results to a specific source population, but rather to isolate the effect of time since last meal on circulating metabolites, internal validity was emphasized over external validity in the present study, as recommended by Rothman and colleagues (79).

5.2.5.2 Information bias

Information bias refers to any systematic differences from the truth, that arises in the collection or handling of data in clinical research (76). In order to minimize the chance of information bias in the present study, well-designed protocols for data collection were

established. SOPs were used for anthropometric measurements, preparations of the standardized breakfast meal, and handling of blood samples, and each procedure were carried out by the same project staff for all participants, in accordance with the applicable protocol. The members of the project staff were responsible for specific tasks regarding data collection, to ensure that all procedures were carried out in the same nature at every study visit.

5.3 Discussion on main findings

In line with the glycostatic hypothesis from the mid 1950s, our results suggest that a rise in blood glucose concentrations coincide with increased feeling of satiety. Mayer postulated that the rise in glycemia after food consumption was the critical factor underlying satiety (50), which correspond well with the positive correlations in the present study between circulating glucose and perceived satiety.

However, despite the fact that the observed fluctuations in blood glucose concentrations correlate well with reported feelings of both hunger and satiety in the current study, it is not possible to state that the glucostatic model account for the coordination of energy intake with expenditure that results in the evolutionary stability of adipose tissues in the long term, based on our results. Limited by the 24-hour study duration, the observed correlations between glucose and self-reported hunger and satiety only represents the short-term aspect of appetite regulation. Hence, further research is required in order to investigated how longer period of food deprivation affected the appetite and regulation of energy balance in the long term.

In line with the results on glucose, insulin concentrations were found to largely correlate with reported hunger, satiety, and desire for food. The strong negative correlation between hunger and circulating insulin concentrations indicates that higher concentrations of insulin correspond to lower reported feelings of hunger, whereas the strongly positive correlation with satiety gives a positive relationship, implying that high concentrations of insulin tend to coincide with high ranks of satiety. Hence, our results, consistent with previous knowledge on the subject, suggests a satiety-promoting effect of insulin as a regulatory hormone.

Even though the results point in the direction of insulin as a hypothalamic energy regulator, it is important to keep in mind that the reported correlations between insulin and appetite only represent the short-term aspect. As emphasized by the results, insulin levels are highly sensitive to the immediate effects of food consumption, which supports the statement that its

primary physiological function is control of glucose homoeostasis, rather than body weight regulation (56).

Despite of increasing concentrations of βHB throughout the intervention, the gMean concentration of 463.54 [2.11] μmol/L at 24 hours do not correspond to what is conventionally considered a state of nutritional ketosis (500–3000 μmol/L). However, considerable variation in measurements is present in the underlying data from three hours postprandially, with the biggest interindividual variation observed at the last blood sampling. After 24 hours of fasting, the maximal circulating concentrations was measured to 1533.3 μmol/L in the present study, which confirms the presence of nutritional ketosis within some participants.

As expected, the mean of perceived hunger decreased in response to the standardized breakfast meal. During postprandial adaptation, the feeling of hunger increased until 8 hours postprandially, before an apparent decrease was observed. From 10 hours into the fasting period, the perceived hunger enters a plateau for the remaining measurements. The changing trend in reported hunger feelings at 8 hours, suggests the onset of the metabolic switch and should be seen in context with the increasing concentrations of ketones. However, due to the nature of the study, it is not possible to state a causal relationship between the decreased feeling of hunger and the increased concentration of circulating ketones. In contrast to these findings, no considerable increase in the feeling of satiety were observed as the concentration of ketones raised, which suggests that the ketones suppress the feeling of hunger, rather than increase the feeling of satiety.

Lastly, the importance of time since last meal when measuring blood concentrations of glucose, insulin and ketones needs to be discussed. Current practice for blood sampling, is to distinguish between fasting and non-fasting blood samples. If to use diagnosis of diabetes as a clinical example, one diagnostic criterion involves measurements of plasma glucose. The cut off is given as two different values depending on whether the sample is fasting or not, in which fasting correspond to not having anything to eat or drink, except form water, for at least 8 hours before the test. The fasting plasma glucose test is usually done first thing in the morning, prior to breakfast, whereas the random plasma glucose test could be conducted at any time of the day, regardless of timing since last meal. As emphasized by the results of this study, concentrations of blood glucose fluctuate within a relatively broad range in the early

postprandial state. Hence, timing since last meal could be of clinical relevance when interpreting the results of random plasma glucose test.

6. Conclusions and future perspectives

Moderate to strong correlations were observed between the biomarkers of interest and the parameters of perceived hunger, satiety, and desire for food. In general, glucose and insulin were associated with less hunger and more satiety, while the opposite was observed for the ketones. However, as the nature of the current study makes it difficult to know whether the measured concentrations and the associated metabolic adaptations gives a representative reflection of the everyday metabolic situation for each subject, more research is needed in order to account for day-to-day variations and strengthen the validity of the results. Multiple factors are likely to influence the metabolic adaptation in the postprandial period, including fluid intake, dietary intake, food choices, state of disease, use of medications, and amount of physical and everyday activity, hence, future research should involve longer study duration with repetitive interventions. Further, to provide data to compare 12 hours of night fasting with 12 hours of day fasting and thus be able to interpret the possible effects of circadian rhythms on postprandial adaptation, a thorough standardization of the last evening meal should be in focus. A longer study duration with an extended period of fasting will also allow participants to enter the state of nutrition ketosis, and hence provide a stronger basis to explore the potential impact of the metabolic switch and ketone production on perceived hunger and satiety.

As the current study only collected data on perceived hunger, satiety and desire for food, which is highly sensitive to the subjective understanding of VAS as a method of assessment, more research is needed on the impact of circulating metabolites on appetite. Further research should supplement the findings with measurements of established hunger and satiety hormones.

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Standard operating procedure					
University of Bergen					
Title	Screening and recruitment	Page	1	Of	3
Version	1	Created on		25.06.2021	
Status	Draft				
Procedure	Screening of participants by phone and arrange date of study visit				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

This standard operating procedure describes the procedures the study team will use for recruiting and screening eligible subjects into a study while following protocol and fulfilling ethical responsibilities for protecting the rights, safety, and welfare of participants and maintenance of a screening log.

2. Scope

This standard operating procedure applies to the activated involved in recruiting and screening participants to the PoMet-study.

3. Responsibility

The individual screening participants by phone is responsible for following this protocol when recruiting and screening participants. The primary investigator is responsible for ensuring that study personnel is well acquainted with the procedure.

Standard operating procedure					
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Version	1	Created on		25.06.2021	
Status	Draft				
Procedure	Screening of participants by phone and agree date of study visit				

4. Procedure

1. Make sure to be logged into the SAFE-server and have the three files: "**Screening log.xlsx**", "**Enrolment log.xlsx**", and "**Participant overview.docx"** available when you call the subject. The files are located in the **Recruitment** folder.

2. Use the file "**Screening_log.xlsx**" in SAFE to find individuals who have expressed their interest in participating in the study by filling out the Skjemaker-form. This file contains a unique "**Screening_ID**" per individual. Change the "Contacted" status to Yes, and find the corresponding row in the file "**Enrolment log.xlsx**" before continuing.
3. Contact subjects by phone. Make sure that you reach the subject directly, not a family member or a housemate. Do not provide further information about the reason for your call to anyone but the intended recipient of the call. Avoid leaving voicemails unless necessary.
4. Introduce yourself properly, provide your name, and that you are working in the project group of "Fastestudien". Confirm that they have expressed their interest in participating in the study and that the subject is still interested in participating. Ask if the they have read the consent form in "**skjemaker**".

Standard operating procedure					
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Procedure	Screening of participants by phone and agree date of study visit				

5. Verify that the participant fulfills all inclusion – and exclusion criteria

Inclusion:

- Born between the years 1991-2001
- BMI between 22-27 kg/m^2 (ask for weight and height to confirm)
- Females: Using the oral contraceptives Melleva, Microgynon, Almina, Loette, Oralcon, or Mirabella.

Exclusion:

- Pregnant or breastfeeding the last three months
- Acute or chronic disease (such as diabetes, thyroid diseases, cancer, cardiovascular disease, inflammatory bowel diseases, etc.) during the last three years
- Celiac disease, lactose intolerance or any other food allergies
- Use of any prescription medications
- Smoking or regular use of other nicotine-containing products ("snus")

6. If the subject fulfills all the inclusion- and exclusion criteria, describe the breakfast meal to the subject:

- 3 small slices of bread, two of them with light cheese and cucumber, and one with jam, and a glass of orange juice

7. Ask if the subject thinks he/she can consume this meal in about 15 minutes.

8. If the subject is **NOT** eligible for inclusion in the study, inform the subject. Document sex (dropdown menu) and the reason for ineligibility in the file **"Enrolment log.xlsx"** in the SAFE-server. Use the dropdown menu in "**Screening log.xlsx**" to indicate that the subject was not enrolled. Save both documents.

9. If the subject **is** eligible for participation in the study, and believes he/she can consume the breakfast meal as instructed, inform the subject that he/she is included in the study.

10. Use the file "**Participant overview.docx**" from the SAFE server to see available study dates, and arrange a date for the study visit. Insert the **Screening_ID** in the table beside the agreed date.

11. Inform the participant that he/she will receive an email with an information letter and a consent form and that the consent form will be signed by the participant at the study visit.

12. Ask if the subject has any questions, and answer any questions that are raised.

13. Fill out the study date in the file **"Enrolment log.xlsx"** in the SAFE server. Use the drop-down menu in the "**Screening log.xlsx**" to indicate that the subject was enrolled. Save both documents.

14. Fill in the agreed study date in "**Infoskriv_før_oppmøte.docx**". Save a PDF copy.

15. Email the participant the files **"Infoskriv_før_oppmøte.pdf"** and **"Samtykkeskriv_v2.pdf".**

15. Fill out all remaining fields in the files **"Enrolment_log.xlsx"** and "**Participant_overview.docx**" in the SAFE-server.

5. Applicable regulations and guidelines

International Conference on Harmonization; Good Clinical Practice: Consolidated Guideline, May 1997

Standard operating procedure					
University of Bergen					
Title	Study visit	Page	1	Of	5
Version	1	Created on		09.07.2021	
Status	Draft				
Procedure	Description of all procedures during the study visit in the PoMet study				
Author	Vegard Lysne				
	Function, name, signature				
Approved by	Åslaug Oddsdatter Matre				
	Function, name, signature				

1. Purpose and background

This standard operating procedure describes the overall procedures and logistics the study team will use during the study visits.

2. Responsibility

The on-site study personell are responsible for following these procedures when organizing the study visit.

The primary investigator is responsible for ensuring that study personnel is well acquainted with the procedure.

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Procedure	Description of all procedures during the study visit in the PoMet study				

3. Procedures

Day 1

1. Take the bread out from the freezer, remove the plastic, and put the slices on a plate and cover in plastic foil.
2. Locate the folders labeled with the correct study IDs. This information is found in the recruitment folder in the SAFE server. The folder contains the study-CRF, lab-CRF, consent form, and VAS scales.
3. Prepare the breakfast meals, cover in foil and place in the refrigerator.
4. Meet the participants at the main entrance at 0730
5. **The participant signs the consent form. Document the time in the CRF**
6. Screen participants to confirm inclusion- and exclusion criteria, enter information directly into the study-CRF
7. Measure height, body weight, and waist circumference, following the corresponding SOPs. Enter the results into the study-CRF.
8. Personnel at FHU administers the venous catheter**. This should happen as early as possible**, as the participant have to wait approximately 10-15 min before the fasting blood sample can be collected. **The above-mentioned measurements (point 6) can be performed in this period.**
9. From the urine sample provided by the participant, distribute urine into 2 x 2 ml Sarstedt tubes with blank cap, and store in freezer at -20 degrees, following the corresponding SOP. Document in lab-CRF.
10. Administer the VAS for hunger sensation.
11. The fasting blood sample is taken. Document in Study-CRF.
12. Transport the blood samples to the laboratory for pre-analytic handling, according to the relevant SOP. Document in the lab-CRF.
13. Serve the breakfast meal, and remind the participant that they have to eat it during approximately 15 minutes. The participants should have a stopwatch to keep track of the time. Note down the start and finish time in the study-CRF. For days when there are two participants, **the second participant should start approximately 7 minutes after the first**, to ensure the logistics with blood sample collection.

Standard operating procedure					
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Procedure	Description of all procedures during the study visit in the PoMet study				

14. After the breakfast meal, follow the **time table (at the bottom)** for data collection, and make sure to follow the relevant SOPs. After the blood sample, ask the participant to fill out the **VAS for hunger sensation** on the designated form found in the participant folder. The participant should not see his/her former VAS-scores. Blood samples are to be transported to the laboratory for pre-analytic handling. Place Sarstedt-tubes consequtively into cardboard boxes in the on-site freezer at –20 degrees. Document everything in the study-CRF and lab-CRF.
15. After the **6h** data collection point, instruct participants to not consume any water until the BodPod measurement is conducted.
16. Between the **8h and 10h** data collection point, start up the BodPod for heating and calibration.
17. After the **10h** data collection time-point, remove the venous catheter, and prepare the participant for the Bod Pod measurement:
    - Instruct the participant to go to the toilet
    - Instruct the participant to change to clothes appropriate for BodPod-measurement (bikini, compression tights, sports bra, underwear, etc.)
18. Conduct body composition analysis using the BodPod, following the relevant SOP. Use the **PoMet ID**, not the participant name, as the identifyer. Document in the study-CRF, and store the printout in the participant folder. If desired, the participant may keep a copy.
19. At the final data collection time-point (12h), a normal blood sample is drawn. Document in the study-CRF. Measure body weight.
20. Participant leaves study center. Remind the participant to only consume water for, and not participate in any strenuous physical activity, and to return to the study center the following morning between 08-0830 for the 24 h sample.
21. Move all frozen Sarstedt tubes and biobank tubes from the temporary storage at –20 degrees to –80 degrees.

Standard operating procedure					
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Status	Draft				
Procedure	Description of all procedures during the study visit in the PoMet study				

Day 2

22. In the morning, welcome the participant. Measure body weight following the relevant SOP. Document in the study-CRF.
23. The 24h blood sample is taken by the FHU personnel, and transported to the laboratory for pre-analytic handling. Document in the Study-CRF and the lab-CRF.
24. Thank the participant for participating, and provide the compensatory gift card. Remind them that they can contact the project group (Vegard or Åslaug) at any time, preferably via email, if they have any questions.

After completion of study visit

25. Locate the 14 Sarstedt tubes with serum in the refrigerator, and the 2 baseline EDTA-blood sample tubes. These are transported by foot to MBF for analysis.
26. Make sure that everything is documented correctly in the study-CRF and lab-CRF.
27. Copy the information from the study-CRF and the lab-CRF into SAFE, in the designated participant folder named with the **PoMEt_ID**. The BodPod result sheet and the consent form are scanned and imported into SAFE for digital storage.
28. Make sure that the physical copy of the study-CRF, lab-CRF, VAS-scales, consent form, and BodPod result sheet are located in the participant folder, and returned to the study folder for physical backup.

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Procedure	Description of all procedures during the study visit in the PoMet study				

4. Time table

Time after meal (min)	Approximate time of day	Procedures	Blood samples	Comment
	0730	Consent form, screening Height, body weight, waist circumference, VAS		
	0800	Install venous catheter Fasting blood sample	1 x serum tube 8 ml 3 x EDTA tubes 3 ml	Extra EDTA tubes
	0815-0830	Breakfast		
15	0845	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
30	0900	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
45	0915	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
60	0930	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
90	1000	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
120	1030	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
180	1130	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
240	1230	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
360	1430	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
480	1630	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	
600	1830	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	Venous catheter is removed
	1830-2030	BodPod Body weight		
720	2030	Blood sample, finger prick VAS	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	Normal blood sample
1440	0830	Blood sample, finger prick VAS Body weight Gift card	1 x serum tube 8 ml 1 x EDTA tubes 3 ml	

Standard operating procedure					
University of Bergen					
Title	Informed consent	Page	1	Of	3
Version	1	Created on		25.06.2021	
Status	Draft				
Procedure	Obtaining informed consent from participants				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

The following procedure should be used when obtaining an informed consent from participant. Obtaining an informed consent is necessary to include a participant in a study. The purpose of this standard operating procedure is to describe the information and essential elements of the study in such a way to a subject, to ensure that the subject's consent is sought in such a way that the subject has ample opportunity to consider whether to participate in the study, under conditions that minimize the possibility of coercion or undue influence. The purpose is also to ensure that freely and voluntarily written informed consent is obtained from each participant in accordance with applicable regulatory requirement and ICH-GCP guidelines.

2. Responsibility

The individual obtaining informed consent is responsible for following this protocol when obtaining the consent. The primary investigator is responsible for ensuring that study personnel are well acquainted with the procedure and have adequate experience and training in performing it.

3. Necessary equipment

2 copies of the consent form (named "Samtykkeskriv_v2») in paper version

Pen

Standard operating procedure					
Title	Informed consent	Page	2	Of	3
Version	1	Created on		25.06.2021	
Status	Draft				
Procedure	Obtaining informed consent				

4. Preparation:

- Make sure that adequate time is provided to provide information about the study to the subject, and that there is enough time to answer all questions raised by the subject.
- Conduct the consent process in a private setting free of coercion and undue influence.

5. Procedure

1. Explain to the subject:

- The aim of the research: To investigate the regulation of metabolites in the hours after a meal, and the adaption to the fasting state.
- Description of the procedures to be followed: Measures of height, weight, waist circumference, BodPod, insertion of a central venous catheter, finger pricks, eating a standardized meal, and fasting for 24 hours (including going home after 12 hours and coming back the next day).
- Any foreseeable risk or discomfort to the subject resulting from participation in the study, including potential dizziness or fatigue due to frequent blood sampling and fasting, and pain in fingers due to dried blood spots.
- That the participant can receive the result from the body composition analysis, and that the participant will receive a gift card worth 400,- NOK to be used at "Morgenlevering".
- That the participant may withdraw from the trial at any time without providing a reason.

2. Ask the subject if there are any questions, and answer any questions that are raised
3. Confirm that the participant understands the study requirements
4. Receive a signature from the subject on both the copies of the consent form
5. Sign both the copies of the consent form with name and date, and document in the Case Record Form (CRF) that informed consent is obtained and at which time.
6. Provide the participant one of the copies of the signed consent, and keep the other

Standard operating procedure					
Title	Informed consent	Page	3	Of	3
Version	1	Created on		25.06.2021	
Status	Draft				
Procedure	Obtaining informed consent				

5. Applicable guidelines

1. Declaration of Helsinki

2. International Conference on Harmonization; Good Clinical Practice: Consolidated Guideline, May 1997

CarbHealth

Procedure Height measurement		Document no.: Version: 1
Written by: Ingrid Revheim	Approved by: Therese Hjort	Date: 2020-11-18

1. PURPOSE AND BACKGROUND

This procedure should be followed when measuring height, with the purpose of ensuring accurate and similar measurements.

2. NECESSARY EEQUIPMENT

The measurements should be conducted using the Seca Stadiometer, model 217 (**Figure 1**).

About the instrument: the mobile stadiometer consists of seven parts that have to be put together. On top, there is a cylinder head that will keep the measurement pole away from the wall and prevent movements that could make the measurements inaccurate.

3. PROCEDURE

Contraindications:

If the participant is unable to stand in an upraised position, the measurement cannot be performed.

Preparation of the participant:

Inform about the procedure and its implication.

Figure 1. Seca Stadiomenter

Conduction of the measurement:

1. The height should be measured without shoes in light clothing to the nearest 0.1 cm.
2. The participants should have their feet gathered, arms along the side, straight legs, and relaxed shoulders. The head must be in the Frankfurt horizontal plane (looking straight ahead, **Figure 2**). Heals, butt, shoulder blades and the back of the head have to be up-close to the vertical measurement pole.
3. The measurement is conducted one time, with maximal inspiration.
4. Make sure to document the measurement in the participant folder.
5. If the participant is not able to stand in an upraised position, one should ask for the height documented in the participant´s passport.

Figure 2. Measurement of height with the correct placement of the head.

Nonconformity and accuracy:

The measurement of height should be performed at the same time of day, preferably the afternoon. In cases where overweight and obesity are an obstacle for placing heals, butt and shoulders up-close to the stadiometer, the participants should be standing up straight.

4. CLEANING

The stadiometer should be washed with a moist cloth or with surface disinfectant (e.g., Antibac).

5. REFERENCES

1. Gibson RS. Principals of Nutritional Assessment. 2nd ed. New York: Oxford University Press; 2005.

6. ATTACHMENTS

Attachment 1: User manual, Seca 217

Attachment 2: Quality control, height measurement

CarbHealth

Procedure Measurement of body weight		Document no.: Version: 1
Written by: Ingrid Revheim	Approved by: Therese Hjort	Date: 2020-11-18

1. PURPOSE AND BACKGROUND

The procedure should be followed when measuring body weight, with the purpose of ensuring accurate and similar measurements.

2. EQUIPMENT

The measurement should be conducted using the Seca scale, model 877 (**Figure 1**). The scale should be placed on a hard surface. A light step on the scale will turn it on. Before asking the participant to step up on the scale for measurements, make sure the display shows 0 kg.

Figure 1. Seca scale, model 877

3. PROCEDURE

Contraindications:

The measurement cannot be performed if the participant is unable to stand on their own and a chair-scale or bed-scale is not available. The maximum body weight that can be measured is 200 kg.

Preparation of the participant:
Inform about the procedure and its implementation. The participants should remove heavy clothing and shoes and empty their pockets.

Conduction of the measurement

1. The measurement is carried out once.
2. Ask the participant to step up on the scale when the display shows 0 kg. Read and document the weight shown on the display.

4. CLEANING

The scale should be washed with a moist cloth or surface disinfectant (e.g., Antibac).

5. CALIBRATION

For calibration of the scale, please contact:

Teck-Ho Serivce AS
E-mail: post@teckhoservice.no
Phone: +47 55 29 22 03/+47 92 06 83 26

6. REFERENCES

1. Gibson RS. Principals of Nutritional Assessment. 2nd ed. New York: Oxford University Press; 2005.

7. ATTACHMENTS

Attachment 1: User manual, Seca 877
Attachment 2: Quality control, scale

Standard operating procedure					
University of Bergen					
Title	Waist circumference	Page	1	Of	3
Version	1	Created on		25.06.2021	
Procedure	Measurement of waist circumference				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

This procedure should be used when measuring the waist circumference,
to ensure accurate and similar measurements.

2. Responsibility

The individual performing the procedure is responsible for following this protocol when measuring waist circumference. The primary investigator is responsible for ensuring that study personnel are well acquainted with the procedure and have adequate experience and training in performing it.

3. Necessary equipment

Measuring tape, Seca 203 measuring tape

Alcohol sanitizer

Standard operating procedure					
Title	Waist circumference	Page	2	Of	3
Version	1	Created on		25.06.2021	

3. Procedure

Contraindications

If the participant is unable to stand in an upraised position.

Preparation of the participant

1. Inform the participant about the procedure.

2. Ask the participant to fold up the t-shirt/sweater, so the measurement can be conducted directly to the skin at the stomach/hip

Implementation of the measurement

1. Mark the lower rib and upper point of the hip bone
2. The waist measurement is conducted horizontally in the middle between these two points using the measuring tape.
3. The measurement is performed on exhalation, while the participant's arms hang down along the side.
4. Measure to the nearest 0.1 cm
5. Carry out the measurement three times
6. Document the number in the CRF

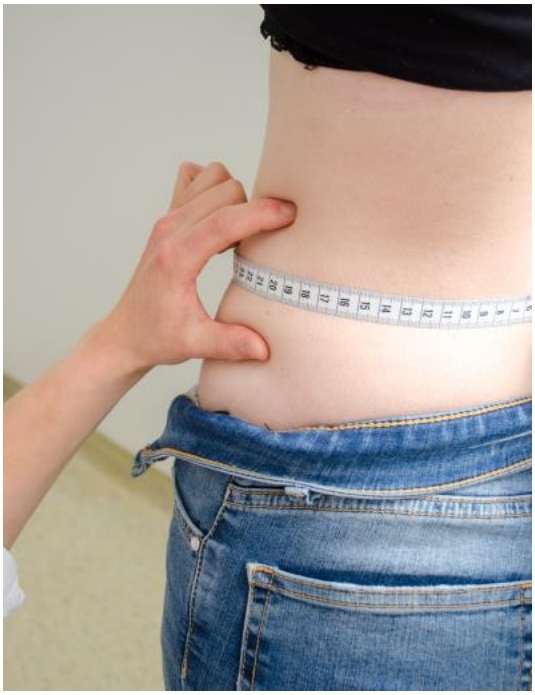

Figure 1 - Correct placement of the measurement tape

Standard operating procedure					
Title	Waist circumference	Page	3	Of	3
Version	1	Created on		25.06.2021	

Cleaning

After conducting the measurement, the measurement tape should be cleaned with a surface disinfectant (*e.g.,* antibacterial sanitizer).

References

1. Gibson RS. Principles of Nutritional Assessment. 2nd ed. New York: Oxford University Press; 2005.

Standard operating procedure					
University of Bergen					
Title	Blood Sampling	Page	1	Of	5
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Handling of blood samples in the PoMet-study				
Author	Vegard Lysne				
	Function, name, signature				
Approved by	Åslaug Oddsdatter Matre				
	Function, name, signature				

1. Purpose and background

This standard operating procedure describes the procedures the study team will use for the handling of blood samples collected during the study visit, including pre-analytic procedures, labeling, transport, and short- and long-term storage. Blood samples are drawn through a venous catheter, and by finger pricking, administrated by trained personnel at the study location.

2. Responsibility

The individual handling the blood samples is responsible for following this protocol. The primary investigator is responsible for ensuring that study personnel are well acquainted with the procedure.

3. Scope

In total, blood samples are drawn at 14 time-points, of which 13 during the first study day and the final blood sample taken the subsequent morning after 24h fasting.

4. Procedures for blood sample collection

Total number of samples per participant

- 14 x 5 ml waste samples
- 14 x 5 ml serum samples
- 14 x 3.5 ml serum samples
- 17 x 3 ml EDTA-blood
- 14 x finger pricks

Total: 233 mL

- Fasting: 1 x 5ml and 1 x 3.5 ml Serum, 3 x 3 ml EDTA
- 24h: 1 x 5 ml and 1 x 3.5 ml Serum. 2 x 3 ml EDTA
- All other time points: 1 x 5 ml and 1 x 3.5 ml Serum, 1 x 3 ml EDTA

Blood samples are drawn from a venous catheter placed in the elbow cavity at baseline. At each sample time-point, the following steps should be followed:

- Flush with 2,5 ml saline solution
- Establish 3-way port
- Draw 2,5 ml from the catheter using either a needle or a waste tube
- Fill the designated tubes
- Flush with 10 ml saline solution after the final tube

The blood samples should be taken within ± 2 min from the designated sampling time-point. At each time-point, the finger prick should be performed first. In case of trouble with blood sample collection, **the serum tube is to be given priority** above the EDTA tube.

If the flow through the venous catheter is discontinued, a new catheter is placed, or a normal blood sample is taken. This decision is to be made by the on-site staff depending on the remaining number of blood samples.

After the 10-hour sample, the venous catheter is removed. The 12-hour sample is taken as a normal blood sample. At the 24h visit, a normal blood sample is taken.

5. Procedure for finger prick for capillary glucose and dried blood spots

At each time-point, before the venous blood sample, one finger is pricked for capillary blood. Capillary blood glucose is measured by HemoCue 201RT, and the result is manually entered into the CRF.

6. Labeling of tubes

All tubes will be labeled with a 9-character code with the format **AAAA_B_CC**, consisting of the PoMet ID (AAAA), Sample type (B), and Sample ID (CC), separated by underscores.

- **PoMet ID**
  All participants will be given a four-character PoMet ID consisting of "PM" (short for PoMet) and a randomly assigned number between 01 and 36. Hence, PoMet ID ranges from PM01 to PM36.
- **Sample type**
  Because the tubes contain different materials, this is also indicated on the label with a two-character code. Serum samples are labeled with "S", and EDTA plasma samples are labeled with "P".
- **Sample ID**
  Because repeated blood samples are collected in the same individuals, each sample is given a two-character Sample ID corresponding to the time-point at which the sample was collected. The sample IDs are numbered from 01 to 14, according to the time-points after the standardized breakfast, as outlined in the table below.

Time since meal	Sample ID
Fasting	01
15 min	02
30 min	03
45 min	04
60 min	05
90 min	06
2h	07
3h	08
4h	09
6h	10
8h	11
10h	12
12h	13
24h	14

Examples:
A baseline EDTA plasma sample in subject PM01:
PM01_P_01

A 6h serum sample in subject PM25:
PM25_S_10

7. Preanalytic handling of blood samples

Serum samples are tilted and stored at the bench for 30 min before centrifuging at 2200 G for 10 min at room temperature. Centrifuging should take place within 1 hour after blood sample collection. The serum is then distributed into 3 Sarstedt tubes (0.5 ml false bottom tubes) with **red** cap (Bevital x 2, Insulin x 1), 1 Sarstedt tube (5 ml MBF tube) with blank cap, and 2 biobank tubes (FluidX 0.7 ml), 500 µl in each tube. The tubes are labeled according to protocol.

EDTA samples are tilted, and 1 tube is centrifugated at 2200 G for 10 min at 4 degrees. Centrifuging should take place immediately after blood sample collection, within 15 min. The plasma is then distributed into 1 Sarstedt tube (0.5 ml false bottom tube) with **purple** cap (Glucagon), and 1 biobank tube (FluidX 0.7 ml), 500 µl in each tube. The tubes are labeled according to protocol. At time-point 01, 2 extra EDTA tubes are taken, and at the timepoint 14, 1 extra EDTA tube is taken. These are stored at room temperature and transported to MBF for analysis of HbA1c and safety parameters.

8. Storage at FHU

At each time-point 3 Sarstedt tubes containing serum, and 1 Sarstedt tube containing EDTA plasma, are temporarily stored in the freezer at -20 degrees. At the end of the day, all samples are moved to -80 degrees, and stored until the end of study. All samples form 1 participant are stored in the same cardboard-box, following the sample map in Table 1. The boxes are labelled with participant ID (PMXX). Morning samples at day 2 (time-point 14) are stored directly in the freezer at -80 degrees, in the same box. In case of missing blood samples or insufficient volume, the priority in the box goes form left to right (prioritize to fill the column to the left).

Table 1: Sample map (9x9)

S1	S10	S1	S10	S1	S10	P1	P10	U1
S2	S11	S2	S11	S2	S11	P2	P11	U1
S3	S12	S3	S12	S3	S12	P3	P12	U2
S4	S13	S4	S13	S4	S13	P4	P13	U2
S5	S14	S5	S14	S5	S14	P5	P14	
S6		S6		S6		P6		
S7		S7		S7		P7		
S8		S8		S8		P8		
S9		S9		S9		P9		

The 14 5ml Sarstedt tubes with blank cap containing serum, one from each sampling time-point, is collected in a rack and stored overnight in the refrigerator (+4 degrees). The 3 extra EDTA tubes collected at the time-point 01 and 14 are stored overnight in room temperature.

The biobank tubes are consecutively registered into LabVantage, and temporarily stored in the freezer at -20 degrees. At the end of the day, the tubes are moved to -80 degrees. Morning samples at day 2 (time-point 14) are stored directly in the freezer at -80 degrees.

The samples are stored in 4 sets of cardboard boxes, each containing a complete set of samples from 5 individuals. The cardboard boxes are labeled with the 5 distinct Participant IDs (PMXX), Sample type (Serum/EDTA Plasma), and for the three serum samples box number (01-03).

9. Transport

The 14 Sarstedt tubes containing serum, and the 3 EDTA tubes from each participant collected at timepoints 01 and 14, are transported to the laboratory each morning after the 24h visit. The blood samples are transported by foot and handed over to a responsible person at MBF.

The frozen serum samples are transported collectively at the end of study. 2 sets of Sarstedt tubes are transported to Bevital (box numbers 01 and 02) and 1 set of Sarstedt tubes (box number 03) is transported to MBF for insulin analysis. (The cardboard box containing frozen EDTA-plasma is transported to MBF for glucagon analysis.) The biobank tubes are collected by the biobank, according to appointments between the biobank and FHU.

Standard operating procedure					
University of Bergen					
Title	Breakfast preparation	Page	1	Of	2
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Preparation of the standardized breakfast meal				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

This SOP describes the procedure to prepare the breakfast meal in the PoMet study. This procedure should be followed to ensure that all participants receive the standardized breakfast meal as similar as possible.

2. Necessary equipment

- Kitchen scale
- Plate
- Glass or cup
- Knife
- Spoon
- Wheat bread
- Light cheese, Norvegia 16% fat
- Butter, Soft flora original
- Strawberry jam, Nora
- Cucumber
- Orange juice, Sunniva original

Figure 1 - The type of strawberry jam, butter, cheese, and orange juice to be used

Standard operating procedure

Title	Breakfast preparation	Page	2	Of	2
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Preparation of the standardized breakfast meal				

3. Procedure

1. Verify that all foods to be used are not expired and that it otherwise looks good (no molds, etc.)

2. Put a plate on the kitchen scale, turn on the scale, and make sure the scale is set to 0 grams

3. Put three slices of bread on the plate. The weight should be 90 grams. Add or remove some bread if needed, to end up with 90 grams of bread.

4. Use the scale to weigh 15 grams of butter and spread the butter on the slices of bread

5. Use the scale to weigh 20 grams of strawberry jam and spread the jam on one slice of bread

6. Use the scale to weigh 40 grams of light cheese and put the cheese on two of the slices of bread

7. Use the scale to weigh 36 grams of cucumber and put the cucumber on the plate

8. Add a glass/cup on the scale and fill it up with 200 grams of orange juice

9. Document in the Case Record Form

Standard operating procedure					
University of Bergen					
Title	Breakfast serving	Page	1	Of	2
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Serving the standardized breakfast meal to the participants				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

This SOP describes the procedure to serve the standardized breakfast meal to the participants in the PoMet-study. This procedure should be followed to ensure that all participants eat the breakfast with the same instructions and under the same conditions.

2. Necessary equipment

1. Prepared breakfast
2. Timer
3. Water

Standard operating procedure					
Title	Breakfast serving	Page	2	Of	2
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Serving the standardized breakfast meal to the participants				

3. Procedure

1. Inform the participant about the content of the breakfast: Three slices of bread, two slices with butter, cheese, and cucumber, one slice with strawberry jam, and one glass of orange juice.

2. Inform the participant that he/she can also drink water with the breakfast if desired

3. Instruct the participant to consume the meal **within 15 minutes** from the first bite

3. Use a timer and start the time when the participant takes the first bite or first sip of the breakfast. In the Case Record Form, register the time when the breakfast meal started.

4. When the last bite or last sip is consumed, stop the timer.

5. In the Case Report Form, register the time the participant finished the breakfast

6. In the Case Report Form, register the number of minutes the participant used to consume the meal

Standard operating procedure					
University of Bergen					
Title	Urine Sampling	Page	1	Of	3
Version	1	Created on		01.07.2021	
Status	Draft				
Procedure	Handling of blood samples in the PoMet-study				
Author	Åslaug Oddsdatter Matre				
	Function, name, signature				
Approved by	Vegard Lysne				
	Function, name, signature				

1. Purpose and background

This standard operating procedure describes the procedures the study team will use for the handling of urine samples collected during the study visit, including preanalytic procedures, labeling, transport, and short- and long-term storage. Urine samples are collected consecutively during the first 12 hours after the breakfast meal.

2. Responsibility

The individual handling the urine samples is responsible for following this protocol. The primary investigator is responsible for ensuring that study personnel are well acquainted with the procedure.

3. Procedures for urine sample collection

The participants are asked in advance to bring a complete morning urine sample. They are given the option of picking up a container at the study center before participation, or they can use a clean container (e.g., a jam jar). After the first day, the participant will bring the container back home for the second morning urine sample.

4. Labeling of tubes

All tubes will be labeled with a 9-character code with the format AAAA_U_BB, consisting of the participant ID (AAAA), U for urine, and Sample number (BB), separated by underscores.

- **Participant ID**
  All participants will be given a four-character PoMet ID consisting of "PM" (short for PoMet) and a randomly assigned number between 01 and 36. Hence, PoMet-ID ranges from PM01 to PM36.
- **Sample number**
  Because morning urine samples are collected both days, each sample is labeled with either 01 or 14, corresponding to the respective time-points.

Examples:

Day 1 morning urine in subject PM01: PM01_U_01
Day 2 morning urine in subject PM25: PM25_U_14

5. Preanalytic handling and storage of urine samples

The urine sample is distributed into two 2mL Sarstedt tubes with blank cap. **Only fill 1.5 ml of urine in each tube.** Store the samples temporarily in the freezer at –20 degrees in the cardboard box belonging to the participant. Store the sample in the box according to the map in Table 1. Excess urine are flushed down in the toilet, and the containers are washed thoroughly. At the end of the day, the cardboard boxes are transferred to the freezer in -80 degrees.

Table 1: Sample map (9x9)

S1	S10	S1	S10	S1	S10	P1	P10	U1
S2	S11	S2	S11	S2	S11	P2	P11	U1
S3	S12	S3	S12	S3	S12	P3	P12	U2
S4	S13	S4	S13	S4	S13	P4	P13	U2
S5	S14	S5	S14	S5	S14	P5	P14	
S6		S6		S6		P6		
S7		S7		S7		P7		
S8		S8		S8		P8		
S9		S9		S9		P9		

1. INTRODUCTION

The following procedure should be used when measuring body composition. BOD POD is considered the gold standard in the measurement of body composition changes over time. It can be used for both adults and children. Estimates for body composition (fat versus fat-free mass) will be completed by applying measures of whole-body densitometry obtained from air displacement plethysmography (ADP) in the BOD POD. The BOD POD also can measure thoracic gas volume (TGV). The method is safe and non-invasive and is therefore suitable for frequent, longitudinal tracking of body composition and metabolic changes over time.

2. RESPONSIBILITY

The individual performing the procedure is responsible for following this protocol when measuring body composition. The primary investigator is responsible for ensuring that study personnel are well acquainted with the procedure and have adequate experience and training in performing it.

3. EQUIPMENT

The measurement is conducted with a BOD POD (Figure 1). Each BOD POD comes with hardware and software required to perform the body composition test. This includes PC with a cart, integrated digital scale, and calibration possibilities.

Figure 1: COSMED BOD POD

4. PROCEDURE

Contraindications:

The measurement cannot be performed if the participant is claustrophobic or have a body weight above 250 kg.

Preparation of the participant:

Inform the participant of the conduction of the measurement. Then inform the participant to but on a swimming cap and bathing suit.

Implementation of the measurement:

1. Measure the subject's body weight and height before entering the BOD POD by standardized procedures.
2. The BOD POD resembles an egg-shaped chamber that includes a window and has air moving in the enclosed space where the subject sits. If the subject begins to feel uncomfortable while enclosed in this chamber, a button located by the subject's knee can be pressed to open the

door. The BOD POD measures the airflow as well as changes in the airflow that occur while the subject is in the chamber.

3. The subject will be instructed to sit quietly while the first measurements of airflow are conducted.
4. The technician will instruct on breathing properly through a tube with the subject using only his/her mouth while the nose is clamped to measure the volume of air in the lungs.
5. The final procedure in measuring the amount of air in the lungs will require that the subject puff three times into the tube as if laughing. The whole test takes approximately 6-8 minutes but maybe longer if the subject has any trouble performing the proper breathing techniques that may need to be repeated.

5. CALIBRATION AND MAINTENANCE

An annual service, support, and maintenance are provided by the distributor Diacor AS.

6. REFERENCES

https://www.cosmed.com/en/products/body-composition/bod-pod

Case record form

Postprandial Metabolism in Healthy Young Subjects

The PoMet-study

Chief investigator: Vegard Lysne

Study ID-number: | | | | |

Subject initials: | | | |

I am confident that the information supplied in this case record form is complete and accurate data.

I confirm that the study was conducted in accordance with the protocol and any protocol amendments and that written informed consent was obtained prior to the study.

Investigator's signature: _____

Date of signature: _____._____._____ (dd.mm.yy)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

Table of contents

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

1. Informed consent

Participant has provided written informed consent	☐ Yes ☐ No
Obtained at:	_____:_____ (time, hh:mm) _____._____._____ (date, dd.mm.yy)
Obtained by:	_____ (investigator)
Signature:	_____ (investigator)
Version of consent form:	_____ (version)
If written informed consent is not provided, the subject cannot participate in the study.	

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

2. Screening

Is the subject born **outside** the years 1991-2001?	☐ No	☐ Yes
Does the subject use "snuff" or smoke regularly?	☐ No	☐ Yes
Is the subject treated with prescription drugs?	☐ No	☐ Yes
Does the subject have a food allergy or intolerance interfering with the standardized breakfast meal (gluten, citrus)?	☐ No	☐ Yes
Has the subject changed weight > 5% the last three months?	☐ No	☐ Yes
Has the subject experienced acute or chronic illness the last 3 years?	☐ No	☐ Yes
Has the subject been pregnant or breastfed the last 3 months?	☐ No	☐ Yes
If the answer is **yes** to any of the questions, the subject must be excluded from the study.		
Does the subject fulfill the criteria for inclusion in the study?	☐ No	☐ Yes

Conducted by: _____ (investigator)

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

3. Anthropometric measures at baseline

Height (cm)	☐ Conducted, measured height: Result: _____ cm (round to nearest 0.1 cm) ☐ Not conducted, self-reported height: Result: _____ cm (round to nearest 0.1 cm)
Weight (kg)	☐ Conducted, measured weight Result: _____ kg (round to nearest 0.1 kg) ☐ Not conducted, self-reported weight Result: _____ kg (round to nearest 0.1 kg)
Waist circumference (cm)	☐ Conducted Result: _____ cm (round to nearest 0.1 cm) ☐ Not conducted Self-reported waist circumference: _____ cm

Measures conducted by: _____ (investigator name)

Measures conducted at: _____:_____ (time, hh:mm)

Signature: _____(investigator signature)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

4. Insertion of venous catheter

A venous catheter was placed	☐ No	☐ Yes
	If <u>yes</u>, which arm?	
	☐ Left	☐ Right
Venous catheter was placed at _____:_____ (time, hh:mm) **Signature**: _____ (investigator signature)		

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

5. The breakfast meal

Preparing the breakfast	Bread: _____ grams Butter: _____ grams Light cheese: _____ grams Strawberry jam: _____ grams Cucumber: _____ grams Orange juice: _____ grams
Breakfast prepared by: _____ (investigator name) **Signature**: _____ (investigator signature)	
Eating the breakfast	Provided at _____:_____ (time, hh:mm) Finished at _____:_____ (time, hh:mm) The participant spent _____ minutes to finish the breakfast.
Provided by: _____ (investigator) **Signature:** _____ (investigator)	

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

6. Standardization questions

Has the subject been taking dietary supplements the last 7 days?	☐ No ☐ Yes **If <u>yes</u>, when and what supplement? Answer here:**
Has the subject been smoking, used "snuff" or any other nicotine containing products the last 7 days?	☐ No ☐ Yes **If <u>yes</u>, when and what? Answer here:**
Has the subject been drinking alcohol or using any drugs the last 24 hours?	☐ No ☐ Yes **If <u>yes</u>, when and what? Answer here:**
Has the subject been doing strenuous physical activity the last 24 hours?	☐ No ☐ Yes **If <u>yes</u>, when and what? Answer here:**
Has the subject been eating or drinking anything other than water since 8PM yesterday?	☐ No ☐ Yes **If <u>yes</u>, when and what? Answer here:**
When was the last meal consumed?	_____:_____ (time, hh:mm) _____._____._____ (date, dd.mm.yy)

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Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

Was the last meal yesterday according to the instructions provided?	☐ No ☐ Yes **If <u>no</u>, what was eaten and when? Answer here:**
Does the subject have menstrual bleedings regularly?	☐ No ☐ Yes ☐ Not applicable (male) **If <u>yes</u>, when was the first day of the last menstrual bleeding? Answer here:** _____._____._____ (dd.mm.yy)

Conducted by: _____ (investigator)

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

7. Participant characteristics

Sex	☐ Male ☐ Female
Date of birth	_____._____._____ (date, dd.mm.yy)
Ethnicity	☐ Caucasian ☐ Asian ☐ African ☐ Other, specify: _____
Weight stability the last three months	☐ Increased weight: _____ kg (round to nearest 0.1 kg) ☐ Decreased weight: _____ kg (round to nearest 0.1 kg) ☐ Stable weight
Use of contraceptives	☐ Not applicable (male) ☐ Does not use any form for contraceptive ☐ Combined oral contraceptives (p-pille) Type: _____ ☐ Progestogen-only contraceptive pill (minipille) Type: _____ ☐ Contraceptive implant (p-stav) Type: _____ ☐ Contraceptive vaginal ring (p-ring) Type: _____

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

	☐ Intrauterine contraceptive device with hormones (hormonspiral) ☐ Intrauterine contraceptive device without hormones (kobberspiral) ☐ Other: _____
Duration of contraceptive use	_____ years and _____ months ☐ Not applicable (male) ☐ Does not use contraceptive

Conducted by: _____ (investigator)

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

8. Blood, urine, hunger, chronotype

8.1 Blood samples

Timepoint	**Time (hh:mm)**	**Blood sample**	**Blood glucose measurement (value)**	**Notes**
01	_____:_____			
02	_____:_____			
03	_____:_____			
04	_____:_____			
05	_____:_____			
06	_____:_____			
07	_____:_____			
08	_____:_____			
09	_____:_____			
10	_____:_____			
11	_____:_____			
12	_____:_____			
13	_____:_____			
14	_____:_____			

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

8.2 Visual Analogue Scale for hunger sensation

Timepoints	**Time (hh:mm)**	**1. Hunger (mm)**	**2. Satiety (mm)**	**3. Desire (mm)**	**Notes**
01	_____:_____				
02	_____:_____				
03	_____:_____				
04	_____:_____				
05	_____:_____				
06	_____:_____				
07	_____:_____				
08	_____:_____				
09	_____:_____				
10	_____:_____				
11	_____:_____				
12	_____:_____				
13	_____:_____				
14	_____:_____				

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

8.3 Urine

Urine day 1 **(baseline)**	**Morning urine sample was provided** ☐ Yes ☐ No **If <u>yes</u>:** Volume: _____ ml **Urine was transferred to Sarstedt-tubes** ☐ Yes ☐ No **If <u>yes</u>:** Time: _____:_____ (hh:mm)
Urine day 2 **(baseline)**	**Morning urine sample was provided** ☐ Yes ☐ No **If <u>yes</u>:** Volume: _____ ml **Urine was transferred to Sarstedt-tubes** ☐ Yes ☐ No **If <u>yes</u>:** Time: _____:_____ (hh:mm)

Signature: _____ (investigator)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

8.3.3 Urinestix

Urine DAY 1				
	Sekund	**Negativ**	**Positiv***	
Glukose (GLU)	30 sek			
Erytrocytter (BLO)	60 sek		**IKKE-hemolysert**	**Hemolysert**
Protein (PRO)	60 sek			
Nitritt (NIT)	60 sek			
Leukocytter (LEU)	120 sek			

Signature: _____

Urine DAY 2				
	Sekund	**Negativ**	**Positiv***	
Glukose (GLU)	30 sek			
Erytrocytter (BLO)	60 sek		**IKKE-hemolysert**	**Hemolysert**
Protein (PRO)	60 sek			
Nitritt (NIT)	60 sek			
Leukocytter (LEU)	120 sek			

Signature: _____

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

8.4 Chronotype

Chronotype form was provided to participant	☐ Provided	☐ Not provided

Signature: _____ (investigator)

9. Removal of venous catheter

Removal of venous catheter	☐ Removed Removed at _____:_____ (time, hh:mm)
Removed by: _____ (name) **Signature:** _____ (investigator)	

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

10. BodPod and weight

BodPod	☐ Conducted Conducted at _____:_____ (time, hh:mm) ☐ Not conducted Reason: _____ **Notes (clothing, piercings, etc.):**
Weight (kg)	☐ Conducted, measured weight Result: _____ kg (round to nearest 0.1 kg) At _____:_____ (time, hh:mm ☐ Not conducted Reason: _____
Conducted by: _____ (investigator) **Signature:** _____ (investigator)	

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

11. Participant leaves study center

The participant left the study center	At _____:_____ (time, hh:mm) the _____._____._____ (date, dd.mm.yy)
Signature Date	_____ (investigator) _____._____._____ (dd.mm.yy)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

12. Participant returns to study center

The participant returned to the study center	☐ No ☐ Yes **If yes, when did the participant return to the study center?** At _____:_____ (time, hh:mm) the _____._____._____ (date, dd.mm.yy)
Has the participant performed any strenuous physical activity since leaving the study center?	☐ No ☐ Yes **If yes, when and what? Answer here:**
Has the participant eaten or drank anything other than water since leaving the study center?	☐ No ☐ Yes **If yes, when and what? Answer here:**
Weight (kg)	☐ Conducted, measured weight Result: _____ kg (round to nearest 0.1 kg) At _____:_____ (time, hh:mm) ☐ Not conducted Reason: _____
Blood sample	☐ Conducted ☐ Not conducted
VAS	☐ Conducted ☐ Not conducted
Urine sample	☐ Provided ☐ Not provided
Signature: _____ (investigator) **Date**: _____._____._____ (dd.mm.yy)	

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

13. End of study

13.1 The participant completed the study

☐ Yes ☐ No

If yes:

The subject completed the study the _____._____._____ (date, dd.mm.yy) at

_____:_____ (time, hh:mm).

Investigator's signature: _____

Date of signature: _____._____._____ (dd.mm.yy)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

13.2 The participant experienced an adverse event and was excluded

☐ Yes ☐ No

If yes:

The subject experienced an adverse event the _____._____._____ (date, dd.mm.yy)

_____:_____ (time, hh:mm).

Explain why and what type of event:

Investigator's signature: _____

Date of signature: _____._____._____ (dd.mm.yy)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

13.3 The participant withdrew from the study

☐ Yes ☐ No

If yes:

The participant decided to withdraw from the study during the study the

_____._____._____ (date, dd.mm.yy) at _____:_____ (time, hh:mm).

Explain, if possible, why:

Investigator's signature: _____

Date of signature: _____._____._____ (dd.mm.yy)

Subject ID				Subject initials			Visit date					
							d	d	m	m	y	y

14. Delivery of gift card

The participant received a gift card at "Morgenlevering", to a value of 400 NOK,- when completing the study	☐ Yes ☐ No **If <u>no</u>, explain why:**

15. Notes