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Postprandial metabolism in healthy young subjects (PoMet)

Prepared by

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Abstract

Introduction: When measuring nutritional biomarkers in blood, recent food intake can greatly impact the concentrations. Hence, it may be important to consider prandial status when interpreting the biomarker concentrations. Current practice involves distinguishing between fasting and non-fasting blood samples. However, metabolite concentrations may change gradually during the postprandial period, meaning the current approach may not be sufficient. The aim of this study was to investigate the dynamic changes in the concentration of nutritional biomarkers during the postprandial and postabsorptive periods. The biomarkers of interest for this thesis include electrolytes, lipids, albumin, folate, and cobalamin.

Method: A total of 36 young, healthy study subjects (Age 20-30, BMI 22-27) were recruited. The subjects attended the study centre after a 12-hour overnight fast. After receiving a standardised breakfast meal, the participants consumed only water for the following 24 hours. Blood was drawn at baseline and 13-time points during the intervention at specified times. The blood samples were transported daily to the laboratory for study-specific analyses. Biomarker concentrations were plotted as a function of time since the standardised breakfast, and the geometric mean time course was superimposed on the individual participant data. To evaluate the absolute agreement of measurements at different time points, intraclass correlation coefficients (ICC) were calculated.

Results: Data from 34 participants (18 males, 16 females), of which one dropped out at time point 7, were included in the final analyses. On average, the male participants were about two years older than the female participants. As expected, there were some differences in height, body mass, waist circumference, fat mass percentage, and resting metabolic rate (RMR) between males and females, but the average BMI was similar between the genders. We observed considerable fluctuations in biomarker concentrations, particularly in the early postprandial state. The largest fluctuations were observed for potassium, phosphate and triglycerides. A steady increase was observed throughout the postprandial and postabsorptive state for folate and cobalamin. During the intervention period, magnesium, albumin, HDL, and LDL were relatively stable. The calculated ICCs in the total population were 0.37-0.68 for phosphate, potassium, magnesium, LDL, albumin, folate, and cobalamin. Triglycerides had an ICC of 0.76 and HDL of 0.82 in the total population. Overall, the intraindividual fluctuations were larger in females compared to males, as represented by lower ICCs.

Conclusion: Large fluctuations were observed in the postprandial period for some of the investigated biomarkers. Other biomarkers demonstrated a steady increase throughout the study period or relatively stable concentrations. This suggests that the prandial status, as reflected by time since the last meal, may be a critical factor in interpreting some, but not all, biomarkers. More considerable variation in biomarker concentrations was observed for females compared to males. The collected data from this study increase our understanding of the postprandial changes in metabolite concentrations, which may have extensive implications both in the clinic and in research. To extend the findings from this study, future studies should aim to examine the postprandial and postabsorptive metabolism in different populations and after meals with different compositions and eaten at other times of the day.

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AIPE	Accuracy-in-parameter-estimation
BMI	Body mass index
CRP	C-reactive protein
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ECLIA	Electrochemiluminescence immunoassay
gCI	Geometric confidence interval
gSD	Geometric standard deviation
gSE	Geometric standard error
gMean	Geometric mean
$HbA1_c$	Hemoglobin A_{1c}
HDL	High-density lipoprotein
HPLC	High-Performance
HuMet-study	Human metabolome study
IDL	Intermediate-density lipoprotein
ISE	Ione-selective electrode
ICC	Intraclass correlation coefficients
LDL	Low-density lipoprotein
MBF	Department of Medical Biochemistry and Pharmacology
MCTQ	Munich ChronoType Questionnaire
MCV	Mean corpuscular volume
MMA	Methylmalonic acid
OxLDL	Oxidised form low-density lipoprotein
PoMet-study	The Postprandial Metabolism study
PTH	Parathyroid hormone
RMR	Resting metabolic rate
SOP	Standard operating procedure
VAS	Visual analogue scale
VLDL	Very-low-density lipoprotein
WHO	World health organization

1.0 Introduction

For interpreting the results of biomarkers in blood samples, especially nutritional biomarkers, prandial status can be challenging. This thesis aims to map the dynamic changes in the circulating concentrations of nutritional biomarkers during the postprandial and postabsorptive periods. The biomarkers of interest include electrolytes, lipids, albumin, folate, and cobalamin.

1.1 Metabolomics

Since scientists first started mapping and sequencing the human genome, new technology has made it possible to obtain a considerable number of molecular measurements within tissues and cells. Metabolomics describes the field of research of small molecules, commonly known as metabolites, within cells, biofluids, tissues and organisms (1). Metabolites are produced during metabolism and can be both substrates and products. Genetic and environmental factors influence a subject's metabolism, and the metabolome directly regulates cellular activity, thus representing the molecular phenotype (2).

Biochemical processes occur continuously within our body, such as absorption in the gastrointestinal tract, metabolisation and distribution of nutrients to target tissues, mobilisation of nutrients and release of signal molecules from cells, degradation and excretion through enterohepatic circulation, colon and kidneys (3). These examples, which are just a selected range of the processes occurring within our body, demonstrate that the concentration of molecules may change rapidly from one time to another. The sum of all these processes determines the concentration of metabolites in cells and tissues at a given time. Importantly, a single measurement of biomarker concentrations provides only a snapshot in time. Unless replicate measurements are done, it is impossible to see how the concentrations change from one time to another.

Metabolomics is a growing field and has received increased interest in recent years. Discovering new biomarkers for complex diseases such as cancer and cardiovascular diseases (CVD) has immense value in detecting and treating these diseases. The growing prevalence of these diseases, correlated to lifestyle, further adds to the importance of the field (4, 5). Analysis of metabolomics in tissues and body fluids such as serum, plasma and urine can give comprehensive information on disease status and development (6). The clinical value of

increased knowledge depends on discovering new and improving the utilisation or increasing robustness of known biomarkers. New knowledge of biomarkers can provide earlier and more accurate diagnostics, prognoses and investigate the effects of new treatments for the individual patient.

1.1.1 Biomarkers

The tremendous development within medical and technological research over the last century has resulted in a spectrum of new biomarkers and new ways to utilise existing biomarkers. This has raised several debates and discussions about biomarkers and finding a good biomarker definition. Today there are several definitions (7).

World Health Organization (WHO) has defined a biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (8). All biomarkers are and can be used to describe former and current health conditions and in screening to predict future health issues. A biomarker can range from a single blood pressure measurement to the quantification of molecules or components in body liquids such as urine and blood. In this thesis we mainly refer to biomarkers quantified in blood or serum.

Within the field of nutrition, biomarkers are used as a target for nutritional status and intake of nutrients. Still, they are also used to detect undiscovered diseases such as inflammations, diagnose metabolic disorders such as diabetes and screen for cardiovascular risk. When evaluating measured concentrations of nutritional biomarkers, it is crucial to know which factors may affect the results and what determines the concentration of a given biomarker at a time.

Evaluation of nutritional biomarkers

The analytical process of a biomarker starts when you decide to take a sample, such as a blood sample, and ends when the measured result comes out. The preanalytical process comprises the preparation before sampling, sample collection, handling and storage of the sample prior to measurement. The sum of preanalytical factors determines the quality of the sample and the reliability of measurement (9). For example, struggling during the collection of a blood sample

may degrade the erythrocytes which leads to hemolysis in the sample. Measurement of potassium or folate in this sample may result in false high circulating concentrations due to the high intracellular concentration of the biomarkers (10). However, most laboratories measure the hemolysis index prior to measuring biomarkers, and different biomarkers have distinct values for the acceptable grade of hemolysis. Therefore, a low-quality blood sample will usually not be considered.

Analytical variation

All laboratory measurements are under the influence of insecurity. The insecurity occurring during the analysis of a sample is known as the analytical variation. This variation comes from random and systematic errors and is known as CV%, a target for precision, and Bias % (11). Most laboratories monitor the analytical variation using quality controls and keeping the analytical variation within acceptable limits. The total variation of an analytical method is determined by the analytical variation and the biological variation.

Biological variation

In vivo, the variation in concentrations of a biomarker is called biological variation (10, 11). Biological variation is defined as random, natural and physiological fluctuations of biomarkers around a homeostatic setting point (12). Biological variation constitutes interindividual- and intraindividual variation (10, 12). The interindividual variation for a biomarker occurs between subjects and forms the foundation for the reported reference values (11). The intraindividual variation occurs within a subject over time, and knowledge of intraindividual variation is essential when comparing replicate measurements of the same biomarker in a subject (11).

Physiological factors

Some biomarkers vary in non-random ways as well. Sex, age and genetic factors are examples of non-random causes affecting the fluctuation in biomarkers. Therefore, the need for different reference values, for some biomarkers, between genders and during various age stages is necessary (12). Previous studies have revealed that genetic factors also are associated with the risk of developing obesity, thus indirectly influencing body composition (13-15). Moreover, the inverse correlation between body composition, in terms of total body fat, and insulin sensitivity consistent with increasing circulating blood glucose concentrations, has further been

demonstrated for more than two decades (16, 17). Thus, it's reasonable to assume that body composition may have an impact on circulating concentrations of biomarkers.

External factors

It's also known that external factors like diurnal variation, food intake, medication, illness, and stress can affect and change the measured concentrations of circulating hormonal and metabolic biomarkers without being necessarily related to pathological metabolic possesses (3, 9, 10). How these external factors influence the measured concentration of biomarkers and how the knowledge of external and preanalytical factors, can be used to verify and explain the measured concentration of the given biomarker, implemented in practice, can be illustrated with a few examples.

The measurement of serum ferritin, a commonly used nutritional biomarker of iron stores, will be influenced by inflammation as this biomarker also is an acute-phase protein. An acute-phase protein will either increase or decrease in response to inflammation (10). In this case, ferritin concentrations will increase, indicating applicable iron stores or suggesting overload. To confirm the measured ferritin status, another biomarker like C-reactive protein (CRP), a different acute-phase protein, can also be measured to verify if the ferritin measurement could have been influenced by inflammation. Another example is the timing of the measurement of blood glucose concentration, as there are several contributors to increased concentration of circulating blood glucose values. For instance, stress induces increased secretion of glucocorticoids, e.g. cortisol, stimulating the breakdown of stored glycogen and inhibiting the uptake and use of glucose in cells, thereby leading to a rise in blood glucose levels (10). Another contributor to an increase in blood glucose values is food intake. The meal's composition will directly affect the acceleration of the raised blood glucose levels and how fast it falls, which again influence insulin secretion (3).

These examples illustrate the understanding of biological variation and how external and preanalytical factors regarding sample collection and metabolic effects are fundamental when evaluating biomarkers in clinics and research. In addition, knowledge of the non-random variation in biomarkers is vital for the correct use of the established reference values, logical interpretation of results, and follow-up and correction of requested tests.

1.2 Metabolism and the transition from the postprandial to the postabsorptive state

The metabolism provides the body with energy for vital processes and synthesis of new organic material to secure development, growth and reproduction. The rate of energy production is determined by a myriad of factors such as sex, genes, body size and composition, which are influenced by age, diseases, diet, and exercise (18). Throughout the day, our metabolism transitions between the postprandial state after a meal and the postabsorptive state. The transition between these two states is a well-regulated process and occurs gradually to sustain homeostasis (19).

1.2.1 Anabolism and catabolism

There are two main pathways of metabolism, catabolic and anabolic (19). Catabolism is a biochemical degradation process where organic molecules are degraded into simple molecules, and energy is released (3). The released energy is utilised for energy-dependent procedures necessary, for instance, for growth, muscle work and digestion. Anabolism is the opposite process in which simple molecules are built up and synthesised into larger, more complex molecules like proteins, lipids, and other molecules building cells and the organism. This process requires energy (3). The primary regulation of the metabolic pathways involves adjusting the catalytic activity of certain enzymes. The regulation must be kept in a balance appropriate for life and growth (3, 19).

1.2.2 Postprandial state

In the postprandial state, dietary components are degraded and absorbed in the gastrointestinal tractus, utilised to refill and increase the body's glycogen- and fat depot. As a response to the rising glucose concentration in the blood after a meal, insulin is secreted from β-cells in islets of Langerhans. Insulin is the primary anabolic hormone, stimulating cellular uptake of glucose, amino acids and lipids. Through glycogenesis, glucose is synthesised to glycogen stored in the liver and muscles. Excessive glucose is converted to lipids in the liver and stored with redundant lipids from the dietary origin as triglycerides in adipose tissue (3, 18). The postprandial phase is an anabolic pathway, where glucose is the primary energy source. The duration of the postprandial state primarily depends on the meal's composition and size. For instance, for a carbohydrate-rich meal, the duration of the postprandial state is shorter than a meal high in fat (3).

1.2.3 Fasting state

When the food is absorbed, digested and stored, the body reaches the postabsorptive state. This state follows the catabolic pathway where blood glucose homeostasis primally relies on glycogenolysis and adipose tissue lipolysis. These processes are mediated by glucagon secretion from α-cells in islets of Langerhans. This primary catabolic hormone increases the breakdown of hepatic glycogen and β-oxidation of fatty acids stored in adipose tissue. Glucagon will also inhibit the synthesis of protein (3). The depletion of glycogen stores initialises the mobilisation of triglycerides and amino acids from skeletal muscles and adipose tissue to provide substrates for gluconeogenesis. The oxidation of fatty acids, primarily in the liver, will accelerate the longer into the postabsorptive fasting state we get, and energy is released through the synthesis of ketone bodies (19-21).

It's clear that the hormonal and metabolic effects in both the postprandial- and fasting states are well studied; The transition from an anabolic pathway, including the rise in insulin secretion and increase in glycogen and fat-reserves, to a catabolic pathway including increased glycogenolysis and lipolysis, increased ketogenesis and activated gluconeogenesis. However, less is known about the effect the transition has on other nutritional biomarkers in a dynamic manner.

1.3 Non-fasting and fasting blood samples

Previous studies have illustrated that nutritional biomarkers will fluctuate due to the changes in metabolic state, especially after a meal (22-24). Therefore, its common practice in both clinic and research to distinguish between non-fasting and fasting blood samples when evaluating these biomarkers. For instance, when performing a glucose tolerance test for diagnosis of diabetes, there are different reference values for fasting and non-fasting serum glucose values (25). The distinction between non-fasting and fasting blood samples depends on the last meal's time and which biomarkers were investigated. The cut-offs are usually 6-12 hours after the previous meal. Fasting blood samples usually also implies no beverage intake except water.

Drawing blood samples in the fasting state may be challenging, particularly in the western part of the world with continuous access to food (26). The blood samples are often drawn in the postprandial period unless patients or study subjects have early doctor visits. It is, therefore,

interesting to know how much a meal can impinge the movements of circulating biomarkers as most studies on metabolism in health and disease have been investigated in the fasting state.

The main challenge with distinguishing non-fasting and fasting blood samples into two exact categories is that there is no clear line for being in one or another. While it may appear convenient to use a binary classification of fasting and non-fasting state, the transition from where homeostasis relies on anabolism to catabolism occurs gradually. This binary classification of a continuous process is therefore arbitrary and can pose some challenges. Since the changes in homeostasis occur gradually, it's reasonable to expect that the changes in underlying biomarkers also change gradually on a continuous scale.

Three studies (27-29) have aimed to describe the dynamics of fasting metabolism and demonstrate that circulating biomarkers fluctuate even after prolonged fasting. However, as data collection started after an overnight fast, these studies do not provide any data on how the biomarkers behave in the postprandial period and adaption to the fasting state. Based on the observed fluctuations of biomarkers during fasting, it's also reasonable to assume that the biomarkers fluctuate even more in the postprandial period.

1.4 Metabolites of focus in this thesis

The aforementioned studies in the previous chapter have described the dynamics of fasting metabolism after an overnight fast. By using a time-course model, we wanted to investigate how electrolytes, transport molecules, lipids, folate and cobalamin fluctuate from the postprandial period and over in the adaption to the fasting state. The following sub-chapters describe the functions of these metabolites, the consequences of imbalance in vivo and the biological variation of these metabolites.

1.4.1 Electrolytes

Electrolytes are a generic term for water-soluble charged substances located either in the intracellular compartment (within cells) or in the extracellular compartment (outside cell membranes) (30). An electrolyte could be either positively charged, known as cation, or negatively charged, known as an anion (3). The electrolytes contribute to a major number of vital processes within our body like nerve impulses, muscle contractions or act as cofactors in

enzymatically mediated metabolic reactions (30). Hormones, kidneys and indirectly the lungs strictly regulate the concentrations of circulating electrolytes (3, 10). The most abundant electrolytes are sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), phosphate (P^-) and calcium (Ca^{2+}). This thesis aims to focus on potassium, magnesium, and phosphate.

Potassium

Potassium is the primary electrolyte intracellularly and quantitatively the most important intracellular cation (3). One of the main functions of potassium is maintaining fluid balance through osmoregulation. Still, the cation is also an important contributor to the membrane potential, essential for the transduction of nerve impulses and muscle contraction (30). Hypokalaemia (low potassium concentrations) and hyperkalaemia (high potassium concentrations) may negatively impact and lead to heart arrhythmia and cause exhaustion. Situations that cause profound loss of electrolytes and fluids such as diarrhoea, vomiting, and use of medications such as diuretics, which increase the excretion of both Na^+ and K^+, are some of the most common causes of hypokalaemia. The leading causes of hyperkalaemia are chronic kidney disease and uncontrolled diabetes (3). The recommended daily intake for potassium is 3.1 g/d (females) and 3.5 g/d (males), and the primary sources of potassium in the Nordic diet are potatoes, fruits, berries, vegetables, milk and other dairy products (31). Postprandially, there is a shift in cellular uptake of dietary potassium until the kidneys excrete the load to maintain homeostasis. The shift is an effect of the anabolic hormone insulin, that mediates an increase in the activity of Na^+/K^+ ATPase (32).

Magnesium

Magnesium is a divalent cation involved in a range of biochemical and physiological reactions within our body, such as energy-dependent membrane transport, gene regulations and neuromuscular transmission (3). Hypomagnesemia and hypermagnesemia can lead to heart arrhythmia and symptoms such as paralysis, dyspnea, dizziness, cramps, and fatigue. The leading cause of hypermagnesemia is reduced excretion from kidneys because of acute or chronic kidney disease. Malnutrition and malabsorption, often in combination with excessive alcohol intake are common causes of hypomagnesemia (3). The daily recommended magnesium intake is 350 mg/d and 280 mg/d for males and females, respectively (31). In the Nordic diet, the main sources of magnesium are green, leafy vegetables, legumes, and whole-grain cereals.

Phosphorus

Around 85% of the total body phosphorus is located in our skeletal as phosphorus ions. The rest is found as complexes intracellularly and as phospholipids in plasma. As a part of the calcium metabolism, circulating phosphorus concentrations depend on the concentrations of parathyroid hormone (PTH) and the activity of 1,25-dihydroxy vitamin D (3). Increased release of PTH results in mobilising phosphorus from the skeletal, which leads to increased circulating concentrations. Increased activity of 1,25-dihydroxy increases intestinal absorption and thereby and increases the circulating phosphorus concentrations (3, 30).

The major cause of high circulating phosphorus concentrations is end-stage kidney disease. Still, conditions such as hypoparathyroidism, acidosis, and hypervitaminosis D can also contribute to high circulating phosphorus concentrations. Low levels can be caused by, for instance, hyperparathyroidism and low levels of vitamin D (3). The recommended daily intake of phosphorus is 600 mg/d and the main sources of phosphorus in the Nordic diet are meat, milk, legumes and grain products (31). After a meal, especially a meal high in carbohydrates, the circulating concentrations of phosphorus will decrease as insulin also stimulates cellular uptake of phosphorus (10, 33). The circulating concentrations of the electrolyte are up to 10-30% higher in the night compared to the morning (10, 34).

1.4.2 Lipids and transport molecules

Lipids are one of the major nutrients, an important source of energy and an essential component at a molecular level, forming the basis of cellular membranes, steroid hormones, bile acid and other signalling molecules. Some fatty acids are essential and must be provided by food as they cannot be synthesised in the body. Lipids tend to be and are usually classified as hydrophobic molecules due to their multiple nonpolar covalent bonds. Consequently, they're more or less not water-soluble and depend on transport molecules such as micelles, chylomicrons, albumin and lipoproteins for digestion and distribution in the circulation (3).

Triglycerides

Triglycerides consist of three fatty acids attached to glycerol by ester bonds and trihydroxy alcohol. The fatty acids are usually diverse, and their composition varies with the origin of the

triglyceride. Triglycerides represent most adipose tissue and are a highly concentrated form of stored energy. Nearly 95% of dietary fat is accounted for as triglycerides, and after a meal, the circulating triglycerides are transported in chylomicrons. Lipoprotein lipase in the peripheral capillary breaks down the triglycerides to glycerol and free fatty acids. The free fatty acids can then be stored as triglycerides in adipose tissue, oxidized by muscle tissue or transported to other tissues bound to albumin. The chylomicron remnant is transported to the liver, where it is removed. Until the next meal, the body depends on triglycerides produced in the liver, which circulates as very-low-density lipoproteins (VLDL), or stored triglycerides in adipose tissue. As lipids are transferred to the tissues, circulating VLDL lipoproteins is converted to intermediate-density lipoproteins (IDL) and then to low-density lipoproteins (LDL), which delivers cholesterol to cells (3).

The circulating concentrations of triglycerides rise after a meal and peaks approximately 2-3 hours postprandially (3, 35). The size of the peak depends on the meal's composition, and a high-calorie meal consisting of fat, especially saturated fatty acids, and carbohydrates will lead to a higher peak. However, previous studies have concluded that non-fasting blood samples are suitable for incipient mapping of cardiovascular risk and lipid status when triglyceride levels <4,5 mmol/L (36-38). Nevertheless, when evaluating the biomarker as a part of follow-up, its recommenced to draw the blood sample in the fasting state (36).

Lack of exercise, use of oral contraceptives and excessive alcohol intake may result in elevated circulating triglyceride concentrations, and during pregnancy, the triglyceride concentrations rise until delivery (10). Genetic defects leading to disordered triglyceride metabolism causes primary hypertriglyceridemia (10, 39). Secondary hypertriglyceridemia can be caused by medical conditions such as diabetes mellitus and hypothyroidism, nutrition or medications (10, 11, 39). Untreated hypertriglyceridemia may lead to pancreatitis.

Lipoproteins

Lipoproteins are complexes of both protein and lipid, allowing triglycerides, phospholipids, cholesterol and cholesterol esters, which are less soluble in water, to be transported in aqueous environments. Lipoproteins consist of a hydrophobic core surrounded by phospholipids, which have a hydrophilic head, and apolipoproteins. The major lipoproteins are chylomicrons, VLDL, IDL, LDL and high-density lipoproteins (HDL) (3). The focus in this thesis is LDL and HDL.

LDL is the major cholesterol carriers in the circulation, delivering cholesterol to cells, consisting of approximately 45% cholesterol, 25% protein, 25% phospholipids, 10% triglyceride, and the characterising apolipoprotein is apolipoprotein B-100 (10). Most cells have receptors for LDL uptake, and the concentration of these receptors are regulated based on the cell's need for cholesterol. For instance, steroid hormone-producing organs such as the adrenal gland, ovaries and testicles and hepatocytes in the liver, producing bile acid, have a greater need of cholesterol and thereby several LDL receptors. However, if there are a short supply of these receptors, LDL will accumulate in the circulation (3). Elevated circulating LDL concentrations are a risk factor for the development of CVD and have been investigated since the middle of the 20th century (40). Accumulation of LDL may lead to interactions with free radicals and create an oxidised form of LDL (OxLDL). The OxLDL activates immunological and inflammatory mechanisms in the vascular endothelium and can lead to the development of arteriosclerosis (41).

High concentrations of LDL are seen in patients with hyperlipoproteinemia. Primary conditions such as familial hypercholesterolemia cause a defect in the uptake of LDL and thereby hyperlipoproteinemia. High concentrations as a consequence of hypothyroidism and kidney disease are classified as secondary hyperlipoproteinemia (37, 42). Elevated values of LDL are also associated with high-fat diets and excessive alcohol intake (3, 37). Conditions such as hyperthyroidism and hepatic injury may lead to low concentrations (10).

HDL is a relatively compact particle consisting of approximately 50% proteins, 30% phospholipids, 17% cholesterol and 3% triglycerides, and characterised for apolipoprotein A-1 (10). In our circulation, circulating HDL facilitates reverse cholesterol transport, transporting cholesterol from the circulation and back to the liver. Excessive cholesterols and phospholipids, from the degradation of chylomicrons and LDL-molecules and from cell membranes, are picked up by HDL and delivered to the liver for excretion. The HDL particle's ability to accept and transport cholesterol is reflected in its size. As the particles are largest just prior to delivery to the liver, a high portion of large HDL in circulation is also associated with decreased risk of development of CVD (3).

Genetic conditions such as familial hyper-alpha-lipoproteinemia, treatment with antiepileptic drugs and estrogens, and moderate alcohol intake may lead to elevated HDL concentrations.

Low concentrations are associated with obesity and hypertriglyceridemia (10). Diseases such as diabetes mellitus, hepatic and kidney failure, hypo- and hyperthyroidism and in some rare, genetic conditions such as familial hypo-alpha-lipoproteinemia and Tangiers disease can also lead to low concentrations (10, 37, 42).

Acute disease and inflammation can influence the measurement of HDL and LDL and result in reduced levels. Therefore, during these conditions it is recommended to await measurement of these biomarkers. After an inflammation, it is recommended to wait three weeks after recovery. For more severe diseases such as stroke and heart attack, it is recommended to wait until three months after recovery (10). Traditionally, blood samples for analysis of HDL and LDL have been drawn in the fasting state. However, recent studies have found that fasting and non-fasting blood samples give similar results for both HDL and LDL (36-38).

Albumin

Around 60% of the plasma proteins are albumins and stands for about 80% of the colloid osmotic pressure in the veins, providing plasma to not leak out from the capillaries and into the tissues (3, 30). The protein is also a transport molecule for various substances such as fatty acids, bilirubin, electrolytes such as Ca^{2+} and Mg^{+}, hormones, metals and drugs. The molecule is manufactured in the liver, and the production rate impinges on nutritional and environmental stressors. Dehydration causes high concentrations of circulating albumin (43). Low concentrations can be caused by several conditions such as increased degradation due to inflammations, reduced synthesis resulting from hepatocyte injury, malnutrition or malabsorption (3). Conditions such as burnt skin and preeclampsia can also result in leakage of albumin into extravascular space or increased loss, thereby lowering the circulating concentrations (43). Pregnancy or being bedridden can lead to 10% reduced albumin concentrations (10). Previous studies have also demonstrated that oral contraceptives lead to lower albumin concentrations (44-46).

1.4.3 Vitamins

Vitamins are organic compounds involved in a range of biochemical and physiological reactions. Vitamins, except vitamin D, cannot be synthesised in the body and must be provided by food. Vitamins are classified into fat-soluble and water-soluble vitamins. This thesis focus on the water-soluble B-vitamins folate and cobalamin, involved in reactions for energy production, nutrient metabolism and haematopoiesis (3).

Folate (B_9)

Folate is a B-vitamin and a generic term for compounds including folic acid and derivates with nutritional properties corresponding to folic acid (3, 47). Folates have several functions. They serve as cofactors in the transport of one-carbon units in the metabolism of amino acids and the synthesis of nucleic acids (3). There are more than 50 types of folates intracellularly, but N^5-methyltetrahydrofolate is one of the essential forms and involved in the remethylating of homocysteine to methionine methionine-homocysteine cycle in the metabolism of amino acids (3, 10, 31). Deficiency of folate can lead to anaemia due to its requirement for normal cell division, and there is probable evidence that deficiency over time can result in neuropsychiatric disruptions such as depression and psychosis (31, 48). Folate insufficiency during pregnancy or conception may lead to neural tube defects in infants (49). The primary sources of folate in the Nordic diet are cereal products (including bread) and vegetables, and the recommended daily intake is 300 mg/day for males and females (400 µg/d for females of reproductive age) (31). Prolonged fasting of more than 1-2 days and vitamin B_{12} deficiency can lead to elevated serum folate concentrations (50).

Cobalamin (B_{12})

Vitamin B_{12}, also known as cobalamin, is a term for cobalt-containing compounds of microbial origin. Cobalamins are synthesised by intestinal microorganisms in animals and the intake of the vitamin is entirely dependent on the amount of animal food products such as meat, liver, dairy products and fish in the diet (47). Low intake can cause deficiency and recommended daily intake of cobalamins are 2 µg/d (31). The biologically active cobalamins in humans are adenosylcobalamin and methylcobalamin. Adenosylcobalamin work as a cofactor in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A, which has an essential role in the Krebs cycle and in the biosynthesis of heme. Methylcobalamin act as a cofactor in the remethylating of homocysteine to methionine in the methionine-homocysteine cycle in the

metabolism of amino acids (3). Lack of cobalamin leads to accumulation of methylmalonate, also known as methylmalonic acid (MMA), another metabolic biomarker on vitamin B_{12} status (3, 51). Vitamin B_{12} deficiency leads to the accumulation of N^5-methyltetrahydrofolate, which may cause elevated s-folate concentrations and a lack of biologically active folate (51). Deficiency may result in a defect in deoxyribonucleic acid (DNA) synthesis and megaloblastic anaemia due to its role in formation and maintenance of myelin sheath. Megaloblastic anaemia is characterised by fewer and larger red blood cells measured as high mean corpuscular volume (MCV) (48). Lack of cobalamin may also lead to neuropathy and psychological disruptions (3, 52).

2.0 Objectives

The main objective is to describe the dynamic changes in the circulating concentrations of nutritional biomarkers during the postprandial and postabsorptive periods, focusing on electrolytes, lipids, albumin, folate and cobalamin. The second objective is to describe the sex-specific differences in these biomarkers.

3.0 Methodology

3.1 Research environment

The Postprandial Metabolism (PoMet) study, an intervention study, was conducted at the Research Unit for Health Surveys, a core facility at the University of Bergen. The study aimed to collect data of the human metabolome after a 12 hour overnight fast and prolonged fasting for 24 hours after a standardised breakfast meal.

3.2 Recruitment of study subjects

In the PoMet study, we aimed to recruit 18 males and 18 females aged 20-30 years and with a body mass index (BMI) between 22-27 kg/m^2. Participants were recruited through social media advertisements (Facebook, Instagram, Twitter), posters in gyms, stores and at the university of Bergen, and snowball sampling.

All the study subjects included in the study should be free of any known chronic or acute illnesses over the last three years and be weight stable over the previous three months (<5% weight change). They should not use prescription drugs (except contraceptives) or have any allergies or intolerances interfering with the standardised breakfast meal served at the study visit. They should not smoke nor use snuff, be pregnant at the study visit, and not been breastfeeding the prior three months before the study visit. Detailed inclusion and exclusion criteria are presented in Table 3.1.

Table 3.1: Inclusion and exclusion criteria for participate in the PoMet-study.

Inclusion criteria	- Age 20-30 years (birth year 1991-2001) - Self-reported body mass index 22-27 kg/m^2 - Stable weight last three months (<5% variation)
Exclusion criteria	- Acute or chronic diseases such as diabetes, cancer, thyroid diseases, cardiovascular disease, or inflammatory bowel disease during the last three years - Celiac disease or other food allergies interfering with the standardised breakfast meal - Regular use of prescription medications except for contraceptives - Smoking or regular use of other nicotine-containing products - Pregnancy - Breastfeeding the last three months

Potential participants were instructed to complete a web-based questionnaire confirming that they were eligible, and were subsequently contacted by one of the researchers who conducted a pre-screening by phone. Participants eligible for inclusion received information and informed consent on email, so they had time to get to know what the study involved and to read through the consent before the study visit. Study subjects were asked not to use any dietary supplements the week before the study visit, abstain from alcohol and avoid strenuous physical activity during the last 24 hours before the study visit. Further, the participants were instructed to eat an evening meal similar to the standardised breakfast meal described in Table 3.2, at 8 p.m. the prior evening. Through these means, the physiological and metabolic conditions were standardised to maximise between-subjects comparability (26).

3.3 Study visit

The following paragraphs describe the study visit and procedures in more detail. A more detailed description of data collection procedures is provided in section 3.4. The events of data collection on the study visit are illustrated in Figure 3.1

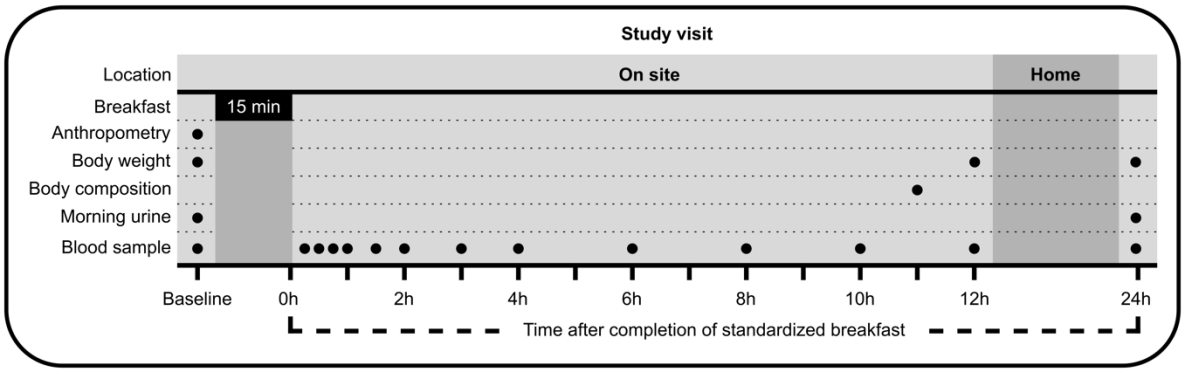

Figure 3.1: Schematic overview of the study visit. The dot represents the timing of the events presented on the left side. The top row indicates the event's location, and below the rows, the timeline describes the number of hours elapsed from the completion of the standardised breakfast.

3.3.1 Arrival and baseline

On the study visit, the participants attended the study centre after an approximate 12 hour overnight fast as they were instructed to have the last meal (Table 3.2) at 8.00 p.m. the prior evening. They were instructed to bring their morning urine and be well hydrated at attendance

to facilitate the insertion of a venous catheter. Upon arrival, the participants were again screened for the inclusion and exclusion criteria, and anthropometric measurements of height (Seca 217), body weight (Seca 877) and waist circumference (Seca 201) were conducted. The body weight was measured with light clothing, and BMI was calculated for screening purposes. Some deviations from the BMI criteria at attendance were accepted due to the variation between scales at home vs study centre, clothing and hydration status. If still eligible for participation, the participants signed the informed consent before continuing the study visit. Then the venous catheter was inserted into the elbow cavity. Ten minutes after insertion and stabilisation of the venous catheter, the baseline blood samples were drawn

3.3.2 Breakfast meal

The study subjects were served the standardised breakfast meal (Table 3.2) and instructed to consume the meal in precisely 15 minutes. They were provided with a countdown timer to ensure compliance. This was done to limit the influence of eating speed on digestion and the release of nutrients into circulation.

The breakfast meal presented in Table 3.2 was assembled with the recommendations from the Norwegian Directorate of Health regarding macronutrients composition (45-60 energy percentages [E%] from carbohydrates, 25-40 E% from fats, and 10-20 E% from protein) and daily energy needs. It was composed to mimic a regular Norwegian breakfast regarding food and macronutrient composition. According to the recommendations from the Norwegian Directorate of Health, a breakfast should pose 20-30% of the daily energy needs, which is estimated to be about 2150 kcal and 2600 kcal per day for inactive females and males, respectively (53). The breakfast contained 520 kcal, 46.3 E% from carbohydrates, 35.9 E% from fats, 16.0 E% from protein, and 1.9 E% from dietary fibre. Based on these estimates, the meal will pose approximately 24% and 20% of daily energy requirements for female and male study subjects, respectively.

Table 3.2: Nutritional content of the breakfast meal in the PoMet-study.

Food	**Amount, *g***	**Energy, *kcal***	**Fat, *g***	**Carbohydrate, *g***	**Dietary fibre, *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 (16%)	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**	**16.0**

3.3.3 Intervention period

After completing the breakfast meal, capillary and venous blood samples were drawn frequently at specified points in time for the next 12 hours postprandially, illustrated in Figure 3.1. Appetite, satiety, and desire were assessed at each time point using a visual analogue scale (VAS). During the study visit, the participants filled out a questionnaire for participant characteristics and a Munich ChronoType Questionnaire (MCTQ).

The study subjects had free access to still water during the study visit, with the exception of the period between 8 and 10 hours postprandially. For practical and logistical reasons, body composition was measured in the evening after the 10 h blood sample, and the participants were instructed to not consume any water during the two hours prior to this analysis. After the blood sampling at the 10th hour postprandially (12th blood sample), the venous catheter was removed, and the body composition calculation was conducted. At 12 hours postprandially, the last blood sample of the day was drawn, and the weight was again measured (Seca 877). The study subjects continued the fasting at home and returned to the study centre the following morning for the final blood samples 24 hours postprandially and measurement of weight (Seca 877). The participants did also bring morning urine the second day. During the intervention, study subjects were instructed to only consumed water and not engage in strenuous physical activity during their time spent outside the study centre.

3.4 Data collection

The following chapter will describe the collected data, how they were collected and handling of these data's during the intervention. The collected data for this thesis include participant characteristics, anthropometric measurements and blood samples.

3.4.1 Characteristics

Questionnaire

Upon arrival, a questionnaire (Appendix 1) was filled out during the study visit for participant characteristics. This questionnaire contained questions to map characteristics such as sex, ethnicity, weight stability over the last three months and use of contraceptives.

Anthropomorphic measurements

Anthropometric measurements were conducted for screening purposes, participant characteristics, and for tracking body weight changes during the intervention. Anthropometric measurements are defined as quantitative, non-invasive measurements such as measurement of height, weight, BMI and waist circumference (54). All the described measurements have been performed at approximately the same time to provide accurate and similar measures.

Body weight

Body mass (kg) was measured at baseline, 12 h and 24 h using a Seca model 877 scale and recorded to the nearest 0.1 kg. It was conducted without shoes in light clothing, empty pockets and carried out once after the display showed 0 kg. The baseline measurement was further used to calculate BMI (kg/m^2) at attendance.

Height

Measurement of height was executed using a Seca 217 stadiometer. The measurement was conducted without shoes in light clothing and to the nearest 0.1 cm. The study subject had their feet gathered, straight legs, arms along the side and relaxed shoulders. The head was in the Frankfurt horizontal plane, looking straight ahead and the heels, butt, shoulder blades, and the head's back close to the vertical measurement pole. A single measurement was carried out, while the participant was maximal inhaling.

Waist circumference

Measurement of waist circumference was performed using a Seca 201 measuring tape to the nearest 0.1 cm. The measure was conducted directly on the skin at the stomach/hip. The hip bones upper point and the lower rib were marked, and the waist circumference was measured horizontally in the middle between the two points. The measurement was performed during exhaling, arms hanging relaxed along the side of the body, and the average of three measurements was recorded.

Calculation of body composition using BOD POD

Body composition was estimated using a BOD POD (Cosmed), which uses an air displacement plethysmograph (ADP) technology to determine body composition. The method is non-invasive and safe, hence suitable for tracking body composition and metabolic changes over time (55, 56). The instrument has an egg-shaped form with a tiny window in front. The BOD POD consists of two chambers. One sealed chamber with a known volume, also referred to as the reference chamber, and a test chamber where the subject is seated (55). The differential pressure between the two chambers is measured, and the relationship to volume can be explained with the Poissons equation for pressure under adiabatic conditions. In other words, the equation describes the relationship between pressure and volume under changing temperature, thereby indirectly finding the body composition (55, 57). By implementing the Siri equation $(\%\, body\ fat = (\frac{4.95}{density}) - 4.50)$, a model based on the two-compartment model where the body is made up of fat mass and fat-free mass, whole-body densitometry is used to assess fat versus fat-free mass (57). The density of fat components is 0.9007 g/cm^3, and the density of fat-free mass is 1.100 g/cm^3 (58, 59).

The study subjects were instructed to wear tight, synthetic underwear or compression clothing of spandex or Lycra type and a swimming cap to displace air pockets in the hair, thereby eliminating volume variations. This is also important for minimising the isothermal air trapped in the fabric and hair since the isothermal air is 40% more compressible than the adiabatic air (57). The baseline measurement of the height was inserted into the software, and body weight was measured before the participant entered the BOD POD. The BOD POD measurements were conducted using the profile "Siri" and predicted thoracic gas volume. The study subjects were informed of the emergency button located by the left knee and to sit still and relax,

breathing normally during the measurements. The instrument conducts two measures of each participant, and the mean value was calculated. The mean value was used for the data analyses.

3.4.2 Collecting and handling of blood samples

In total, there were 14 points in time for blood sampling in the PoMet-study. All samples were collected by trained and certified personnel at the study location. At each point in time, capillary blood glucose was first measured (HemoCue® 201 RT) before the venous blood samples were drawn. For the first 12 time points, the venous blood samples were drawn from a venous catheter. At 12 h- and 24 h postprandially, the two last points in time, were regular venous blood samples. If for any reason, blood sampling from the venous catheter failed, the study personnel and the participant made a joint decision of whether to insert a new catheter or to switch to regular venous blood samples for the remainder of the study visit. The decision depended mainly on participant preferences, as well as the number of remaining blood samples. In total, 11,5 mL of blood were collected at each time point (an additional 5 mL waste blood was drawn from the venous catheter), distributed into serum tubes (8.5 mL, BD Vacutainer® SSTTM II) and EDTA-tubes (3 mL, Vacuette® K2EDTA). At baseline and last time point, an additional two- and one extra 3 mL tube of EDTA blood was drawn for additional analyses.

After collection, serum tubes were stored at room temperature for 30-60 minutes, then centrifuged at 2200xG for 10 minutes at 20ºC. The EDTA tube was centrifuged within 15 minutes after collection, at 2200xG for 10 minutes at 4ºC. After centrifugation, both serum and EDTA-plasma were aliquoted into secondary tubes and temporarily stored in a freezer at -20ºC. At the end of the day, the aliquots of serum and EDTA-plasma were transferred to -80ºC freezer until the end of study. One aliquot of serum from each time point was held in the refrigerator at +4ºC and was daily transported to the central laboratory of Haukeland University Hospital Bergen, together with the additional EDTA-blood collected at baseline and the 24 h time point.

3.5 Quantification of metabolites

All clinical-chemical analysis, except the measurement of glucose quantified using a handheld device (HemoCue® Glucose 201 RT), was performed at the department of Medical Biochemistry and Pharmacology (MBF) at Haukeland University Hospital Bergen, Norway (certified NS-EN ISO 15189:2012).

An automatic biochemistry analyser (Cobas 8000) was used for the quantification of all metabolites except Hemoglobin A_{1c} (HbA1c), which was quantified using High-Performance Liquid Chromatography (HPLC) using a cation exchange column (D-100 system 1, Bio-Rad) in EDTA-blood. Cobas 8000 consist of an ion-selective electrode (ISE) module used for the analysis of serum potassium, a module used for photometric analysis of CRP, creatinine, magnesium, phosphate, HDL, LDL, triglycerides and albumin in serum, and a module for electrochemiluminescence immunoassays (ECLIA) used for quantification of cobalamin and folate in serum. To ensure a healthy study population during the intervention, analysis of HbA1c, glucose, CRP and creatinine were measured in the baseline blood samples for routine clinical data. In this way, we were able to control for undiscovered diseases such as inflammation and metabolic disorders

Table 3.3 lists biomarkers of interest and those measured for routine clinical data, its analysis method, reference values and reported analytical and biological variation. All reference values reported in this thesis are for adults between 20 and 30 years, and the reported reference values and the analytical and biological variation in Table 3.3 are found on www.analyseoversikten.no. The following sub-chapters will shortly describe the analytical principles for quantification of biomarkers presented in Table 3.3.

Table 3.3: Clinical-chemical analysis of biomarkers, its analysis method and the reference values.

Biomarker	Analysis method	Reference values*		Analytical variation (%)	Biological variation (%)
		Males	Females		
HbA1c, mmol/mol	HPLC	<48[1]	<48[1]	3.0	-
Potassium, mmol/L	Indirect ISE	3.5 – 5.0	3.5 – 5.0	2.0	4.8
Glucose, mmol/L	Photometry	4.0 – 6.0[2]	4.0 – 6.0[2]	-	6.1
CRP, mg/L	Photometry	<5	<5	5.0	-
Creatinine, mmol/L	Photometry	60 – 105	45 - 90	3.0	4.5
Magnesium, mmol/L	Photometry	0.71 – 0.94	0.71 – 0.94	2.5	3.6
Phosphate, mmol/L	Photometry	0.75 – 1.65	0.85 – 1.50	3.0	8.5
HDL, mmol/L	Photometry	0.8 – 2.1	1.0 – 2.7	3.0	5.8
LDL, mmol/L	Photometry	1.8 – 5.7	1.8 – 5.7	2.5	8.3
Triglycerides, mmol/L	Photometry	0.45 – 2.60	0.45 – 2.60	3.0	20
Albumin, g/L	Photometry	39 – 50	39 – 50	3.0	8.5
Folate, nmol/L	ECLIA	>9[3]	>9[3]	10	24
Cobalamin, pmol/L	ECLIA	175 - 700	175 - 700	7.0	5.0

Abbreviations: HbA1c, Hemoglobin A_{1c}; CRP, C-reactive protein; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; HPLC, High-Performance Liquid Chromatography; ISE, ion-selective electrode; ECLIA, electrochemiluminescence immunoassay.

1: Values above the reported reference value for HbA1c are the diagnostic criteria for diabetes.

2: Fasting glucose

3: Folate has no reference value, instead there are a medical decision level for deficiency <10.

**Reference values for adults between 20-30 years cited from www.analyseoversikten.no .*

3.5.1 High-Performance Liquid Chromatography (HPLC)

HPLC separates in high resolution using high pressure to force a solution through a packed column of tiny particles, called the stationary phase. A cation exchange column, used to quantify HbA1c, has a negatively charged surface in the stationary phase, allowing attraction with sample cations in the mobile phase. The mobile phase is a buffer solution that transports the sample through the column. The cation exchanger column favour binding of ions of a higher

charge; the higher charge, the longer it will take to elute. As a result of different degrees of affinity to the stationary phase in the column and the mobile phase, distinct components in the sample, in this case, different hemoglobin fractions, will be separated (60). The separated fractions will migrate through the flow cell and reach a filter photometer where the absorbance is measured at a wavelength of 415 nm. The absorbance is also measured at a wavelength of 690 nm to reduce noise.

3.5.2 Ion-selective electrode (ISE)

ISE is an analytical technique based on potentiometry. The principle of potentiometry uses the measurement of the electrical potential (the voltage) between an outer reference electrode and an indicator electrode to determine the activity of ions in a solution (60). The indirect ISE method dilutes the sample before measurement and provides easier maintenance between samples.

The indicator electrode consists of a reference solution of the selective ion, in this case, potassium, and an inner reference electrode. The reference electrodes have a known concentration and a constant cell potential. There is a thin glass membrane at the bottom of the indicator electrode, working as an ion exchanger, allowing the selective ion, potassium, to bind to the electrode selectively. This creates a potential (V) between the indicator electrode and the reference electrode and is measured with a voltmeter. Using the Nernst equation, which says that the ion activity (V) is proportional to the logarithm of the investigated ion, the concentration of the ion, potassium, in the sample is determined (60).

3.5.3 Photometry

By combining assays such as colorimetric, enzymatic or turbidimetric assays and a photometer, it is possible to determine the concentration of a chemical element or compound, representing the biomarker, by comparing the changes in the colour of the reaction mixture. When you send electromagnetic energy, such as light, through a reaction mixture, it's either absorbed (taken up) or transmitted (emitted). A photometric measurement measures the absorbance of the transmitted light in a reaction mixture, and a biochromatic photometric measurement simultaneously measures the absorbance at two wavelengths, compensating for interference. As the absorbance is directly proportional to the concentration of the biomarker, it's possible to determine the concentration using a standard curve made by a calibrator with a known

concentration of the chemical element/compound (60). Which assays are used for quantification of the various biomarkers and at which wavelength (nm) the absorbances are measured at is listed in Table 3.4.

Table 3.4: Assays used to quantify biomarkers measured with a bichromatic spectrophotometer.

Biomarker	**Principles of analysis**	**Wavelength (nm)**	
		Primary	Secondary
C-reactive protein	Immunoturbidimetric assay	570	800
Creatinine	Enzymatic assay	546	700
Magnesium	Colorimetric end-point assay	600	505
Phosphate	Molybdate UV assay	340	700
Albumin	Colorimetric assay	570	505
Triglycerides	Enzymatic colorimetric assay	505	700
High density lipoprotein	Homogenous enzymatic colorimetric assay	600	700
Low density lipoprotein	Homogenous enzymatic colorimetric assay	600	700

Abbreviation: UV, Ultraviolet

Cited from Roche diagnostics

Glucose was quantified using a handheld device, HemoCue® Glucose 201 RT. This method is also based on photometry which measures glucose in hemolysed whole blood after a modified glucose dehydrogenase reaction. However, this handheld device is not as accurate and precise as an automatic biochemistry analyser such as Cobas 8000.

3.5.4 Electrochemiluminescence immunoassay (ECLIA)

The ECLIA method used to measure biomarkers for folate and cobalamin is a competitive immunological method. In this method, a reaction mixture consisting of ruthenium-marked antibodies and sample serum is added to a test cuvette, where the antibodies are in excess. The reaction mixture is incubated, and the antibodies will bind to the biomarker in the sample, creating antigen-biomarker complexes. The number of formed complexes depends on the concentration of the biomarker in the sample; the higher concentration, the more complexes are formed. In the next step, a biotin-marked antigen which mimics the biomarker, and streptavidin-coated microparticles are added to the test cuvette. This new antigen, mimicking

the biomarker, will create complexes with the redundant antibodies from the first incubation. These new complexes, consisting of redundant antibodies and biotin marked antigen, will bind to the streptavidin-coated microparticles. Because of the high affinity between biotin and streptavidin, only biotin marked antigens will bind to the microparticles. The reaction mixture is then transferred to the measuring cell, where excessive antibody-biomarker complexes are washed away, and the microparticle complexes remain. The ruthenium on the microparticle complexes will excite in the measuring cell, creating a luminescence (light) signal. This signal is inversely proportional to the concentration of the biomarker in the sample. Therefore, a high concentration of biomarker will result in a low signal, and a low concentration of biomarker results in a strong signal (61).

3.6 Ethical considerations

The research group has relevant experience with clinical studies and formal training in Good Clinical Practice. Regional Committees for medical and health research ethics have approved the study (REK 236654), and it was registered at the database ClinicalTrials.gov (NCT04989478). All procedures described in this thesis have been described in standard operating procedures (SOPs) (Appendix 2) developed based on locally existing SOPs. The principles of the Declaration of Helsinki have been applied during the intervention.

3.7 Statistics

All statistics in this thesis will be performed using R version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria), and the packages within the Tidyverse (62), and *irrICC*.

3.7.1 Calculation of purposed sample size

The study aims to precisely estimate the changes in metabolites over time and not to test a specific hypothesis. Therefore, rather than a traditional power calculation, the proposed sample size for the intervention was calculated using the accuracy-in-parameter-estimation (AIPE) approach, focusing on the precision of the estimates (63, 64). By specifying the desired maximum width of the confidence intervals, it's possible to estimate the needed sample size using this model.

To achieve the desired multiplicative margin-of-error, i.e. geometric standard error (gSE) 1.96 < 1.10, corresponding to a gSE < 1.05, for at least 80% of the measurements, the freely available data from the Human Metabolome (HuMet) Study was used (27). By using the results from 132 metabolites across 56 time points (7392 estimates) across different metabolic challenges, the observed median (80th percentile) had a geometric standard deviation (gSD) of 1.24 (<1.32). The proposed sample size was found by using the formula $gSE = gSD^{\frac{1}{\sqrt{n}}}$ and solving for *n* in the equation.

$$1.32^{\frac{1}{\sqrt{n}}} < 1.05$$

$$32.4 < n$$

Since the test subjects in HuMet-study were exposed to external factors like stress, which could be expected to increase variation relative to this study, 32 were chosen as a suitable sample size. However, to take into account an expected dropout of 10% due to adverse events regarding the collection of blood samples, a total of 36 participants were recruited to achieve the goal of collecting complete data for 32 study subjects.

3.7.2 Baseline characteristics

The study population's baseline anthropometric and routine clinical data are presented in tables. Tables for baseline anthropometric data describe mean values and its standard deviations for the whole study population and according to sex. The tables also show minimum and maximum measures and the dispersion of the data using 25%, 50% and 75% percentiles. The reported body weight and calculated BMI presented in the table for baseline anthropometric data use the measurement of body weight measured during body composition calculation. This scale was calibrated before each analysis, thus considered a more accurate scale.

Since most biomarkers are skewed with a longer tail towards higher values, as well as negative values not being possible, all biomarker concentrations have been log-transformed before statistical analysis as recommended (65). The table for routine clinical data describes the geometric mean (gMean) and gSD for the whole study population and according to sex. The table also show the dispersion of the data using 25%, 50% and 75% percentiles.

3.7.3 Time-resolved presentation

The main results from the study are presented visually, by plotting all measured metabolite concentrations at each time point as a function of time since the standardised breakfast meal. In the graphs, the gMean metabolite concentrations and the 95% geometric confidence interval (gCI) are superimposed on the raw individual participant data.

3.7.4 Intraclass correlation coefficients (ICC)

To evaluate the degree to which a breakfast meal influences the quantification of biomarkers, the intraclass correlation coefficients (ICCs) have been calculated on the log-transformed data and are presented in a table. The ICCs were calculated on the basis of a two-way random-effects model for absolute agreement, consistent with ICC (2,1) (66).

The ICC is a number with a value between 0 and 1, and the level of reliability is commonly classified into poor, moderate, good and excellent. It is suggested that ICC values < 0.50 indicate poor reliabilities, values between $0.50 \leq$ and < 0.75 indicate moderate reliability, values between $0.75 \leq$ and < 0.90 indicate good reliability and values of ≥ 0.90 indicate excellent reliability (66).

4.0 Results

4.1 Recruitment of study subjects

A flowchart of the recruitment process is illustrated in Figure 4.1. A total of 49 individuals were recruited for pre-screening over the phone for the intervention. Of those, 47 met the requirements listed in Table 3.1 and were considered eligible for participation. Eleven participants were not available at the study dates or withdrew before their visit, and we ended up with a total of 36 individuals, 18 males and 18 females, included in the study. During the study visit, three participants withdrew due to difficulties with blood sampling. Of the non-completing participants, one completed the first two hours of the intervention, and the collected data is included in the results. The remaining two excluded participants had to withdraw on the second time point of blood sampling and were excluded from the analyses. Thus, a total of 33 subjects, 18 males and 15 females, completed the study, but the total number of participants included in the results is 34 individuals.

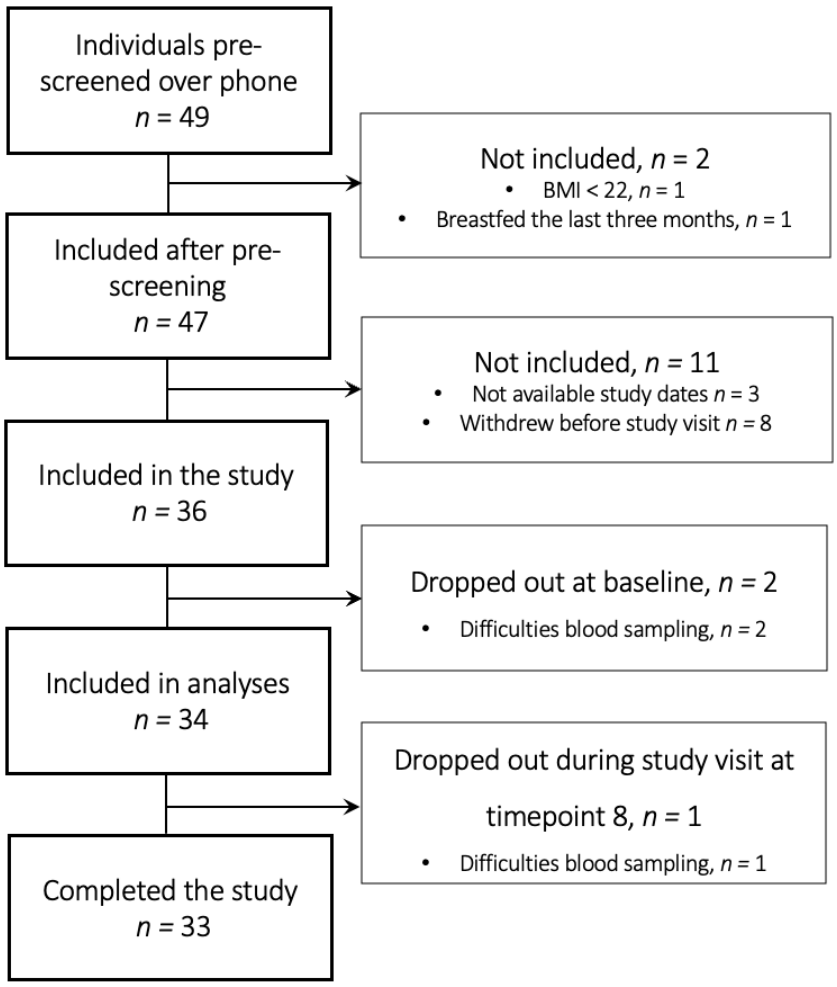

Figure 4.1: Flowchart of the recruitment and inclusion process in the PoMet-study.

4.2 Characteristics of study subjects

The main baseline characteristics of the participants and distribution of these data are provided in Table 4.1. On average, the male participants were about two years older than the female participants. As expected, there were some differences in height, body mass, waist circumference, fat mass percentage, and resting metabolic rate (RMR) between males and females, but the average BMI was similar between the genders. Furthermore, 25% of the population were under the self-reported BMI criteria of 22.0 kg/m^2.

Table 4.1: Baseline anthropometric data (mean ± standard deviation) and the distribution of these data of the study population in PoMet-study.

Parameter	**Mean ±SD**			**Percentiles** *(total population, n = 34)*				
	Male (*n = 18*)	**Female** *(n = 16)*	**Total** *(n = 34)*	**Min**	**25 %**	**50%**	**75%**	**Max**
Age, year	26.2 ± 2.8	24.6 ± 3.0	25.5 ± 3.0	20.0	22.9	25.8	28.0	30.4
Hight, cm	184.4 ± 5.5	168.0 ± 7.6	176.6 ± 10.5	152.5	169.2	178.3	184.8	194.7
Body mass, kg	81.3 ± 8.0	65.5 ± 6.0	74.1 ±10.7	57.2	63.4	74.8	80.0	98.3
BMI kg/m²	23.9 ±1.7	23.1 ±1.5	23.5 ± 1.6	20.2	22.0	23.5	24.8	26.9
Waist circumference, cm	88.3 ± 6.8	74.7 ± 4.3	81.9 ± 8.9	67.3	76.6	80.5	88.9	100.0
Fat mass, %	19.4 ± 7.4	29.0 ± 5.3	23.8 ± 8.1	10.5	15.7	24.9	30.0	41.9
RMR, kcal	1747 ± 163	1277 ± 145	1533 ± 283	1053	1270	1552	1741	2200

Abbreviations: BMI, body mass index; RMR, resting metabolic rate estimated using BOD POD.

The data in Table 4.2 provide the baseline routine clinical measurements. All included subjects had baseline measurements for all biomarkers, within the reference values reported in Table 3.3, presenting the laboratory's specified reference values.

Table 4.2: The baseline clinical data of the study population in PoMet-study.

Biomarker	gMean (gSD)			Percentiles *(total population, n = 34)*		
	Male (*n = 18*)	**Female** *(n = 16)*	**Total** *(n = 34)*	**25%**	**50%**	**75%**
HbA1c, *mmol/mol*	31.8 (1.10)	30.7 (1.11)	31.3 (1.11)	29.3	32.0	34.0
Glucose, mmol/L	5.40 (1.09)	5.12 (1.10)	5.27 (1.10)	4.90	5.20	5.68
CRP *mg /L*	0.57 (2.05)	1.38 (2.62)	0.86 (2.57)	0.50	0.91	1.00
Creatinine, *mmol/L*	81.2 (1.18)	68.2 (1.16)	74.8 (1.19)	69.3	74.5	81.0
Potassium, *mmol/L*	4.06 (1.08)	4.07 (1.07)	4.07 (1.07)	4.00	4.10	4.20
Magnesium, *mmol/L*	0.82 (1.06)	0.81 (1.05)	0.81 (1.06)	0.78	0.82	0.84
Phosphate, *mmol/L*	1.08 (1.14)	1.20 (1.09)	1.14 (1.13)	1.07	1.15	1.22
HDL, *mmol/L*	1.45 (1.20)	1.66 (1.26)	1.55 (1.24)	1.30	1.50	1.78
LDL, *mmol/L*	2.58 (1.39)	2.44 (1.21)	2.51 (1.31)	2.00	2.50	3.18
Triglycerides, *mmol/L*	0.90 (1.46)	0.81 (1.46)	0.85 (1.46)	0.63	0.81	1.17
Albumin, *g/L*	46.6 (1.04)	43.9 (1.06)	45.3 (1.06)	43.3	46.0	47.8
Folate, nmol*/L*	14.0 (1.44)	13.6 (1.36)	13.8 (1.40)	10.8	13.6	16.8
Cobalamin, *pmol/L*	330 (1.44)	325 (1.44)	328 (1.43)	254	317	410

Abbreviations: gMean, geometric mean; gSD, geometric standard deviation; HbA1c, Hemoglobin A_{1c}; CRP, C-reactive protein; HDL, High-density lipoprotein; LDL, Low-density lipoprotein.

4.3 Time-resolved changes in postprandial biomarker concentrations

Serum concentrations of each biomarker of interest (potassium, magnesium, phosphate, albumin, triglycerides, HDL, LDL, folate and cobalamin) have been plotted individually as a function of time since the standardised breakfast, presented in Figures 4.2 - 4.10. Due to hemolysis, some measurements of potassium and folate have been excluded from their respective timelines.

4.3.1 Electrolytes

Potassium

Figure 4.2 graphically presents the measured concentrations of potassium. At an intraindividual scale, there were fluctuations of potassium for the first two hours postprandially. The gMean line initially showed a slight rise in the circulating concentrations of potassium, and 15 minutes after completion of the standardised breakfast meal, the circulating concentrations tended to drop. Then, one hour after completion of the meal, it increased. Furthermore, the concentrations of the potassium peaked approximately 3-4 hours after finishing the meal. Around 6 hours after completing the breakfast meal, the circulating concentrations had returned to the baseline level. After which, the biomarker concentration fell and reached the lowest level after approximately 12 hours. On the gMean timeline, the circulating concentrations were back to baseline levels (Table 4.2) when participants returned for the last blood sample at 24 hours. Comparing the maximum (4.33 mmol/L) and the minimum (3.84 mmol/L) gMean concentrations in the study period there was approximately 0.5 mmol/L difference.

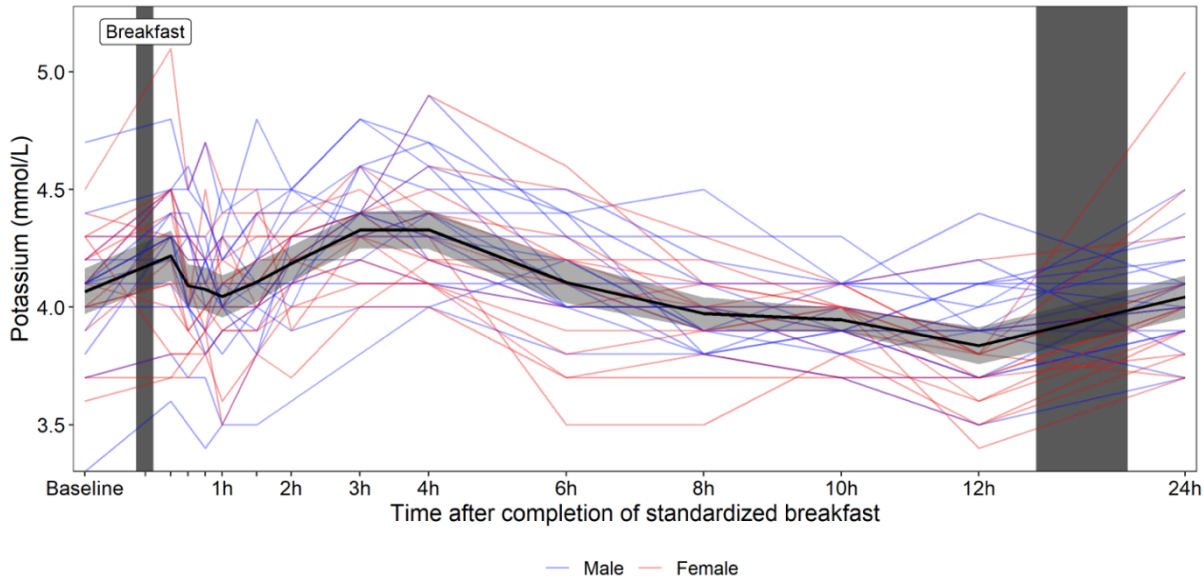

Figure 4.2: ***Quantified serum potassium (mmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre. Note that, due to hemolysis, three measurements have been excluded from the individual timeline; two of the excluded measurements were within the same male participant (the 1st and 10th -time sample) and the 11th point in time in another male participant.*

Magnesium

Time course presentation of the measured magnesium concentrations is displayed in Figure 4.3. For the first two hours of the intervention, there were small fluctuations in the biomarker at an intraindividual scale. The baseline concentrations range from 0.73 mmol/L to 0.91 mmol/L, indicating the interindividual variation of the biomarker. Following the gMean line, the biomarker was quite a stabile the first hour of intervention before it slightly increased until approximately 4 hours postprandially. However, comparing minimum concentrations (gMean 0.81 mmol/L) to the maximum (gMean 0.84 mmol/L), there was a difference of 0.03 mmol/L, indicating virtually no change in circulating concentrations.

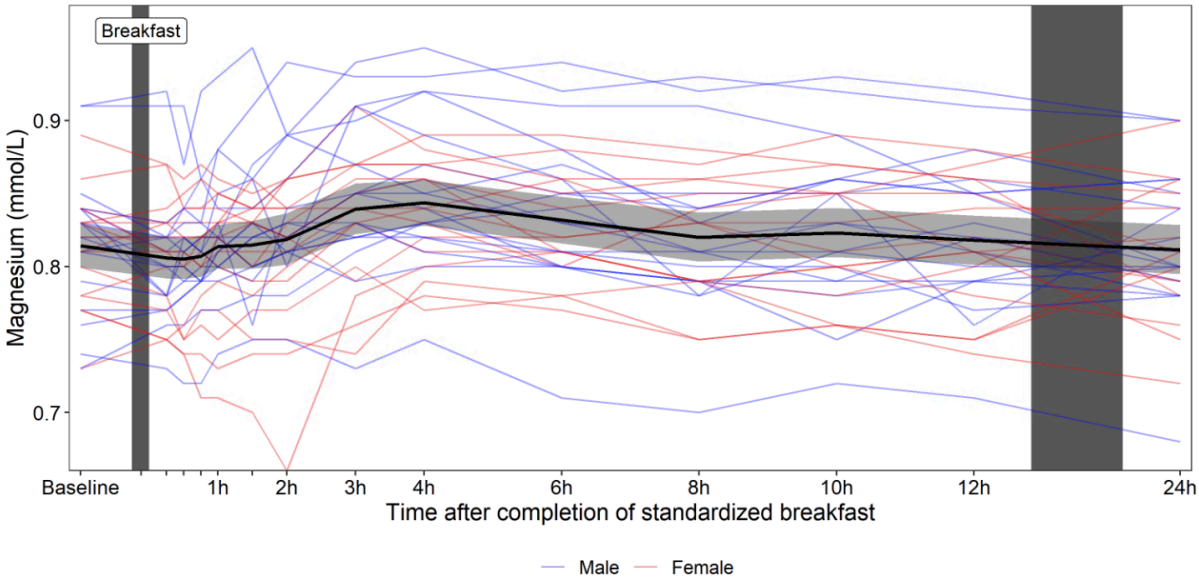

Figure 4.3: ***Quantified serum magnesium (mmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

Phosphate

The measured concentrations of phosphate plotted as a function of time are displayed in Figure 4.4. From baseline and approximately 1.5 hours postprandially, the circulating phosphate concentration decreased and reached the lowest concentrations (gMean 0.99 mmol/L). Then the concentrations increased and reached the peak concentration at around 6 hours (gMean 1.26 mmol/L). There was a slight decrease in the circulating concentrations before it tended to stabilise after 12 hours postprandially. Overnight, the phosphate concentrations increased, and at the last blood sampling 24 h after the meal, the concentrations were higher compared to baseline. By comparing the maximum concentration to the minimum concentration in the gMean timeline, an 0.27 mmol/L difference between the average highest and lowest values was observed. On an intraindividual level, the circulating concentrations of phosphate mainly followed the same pattern as the gMean line.

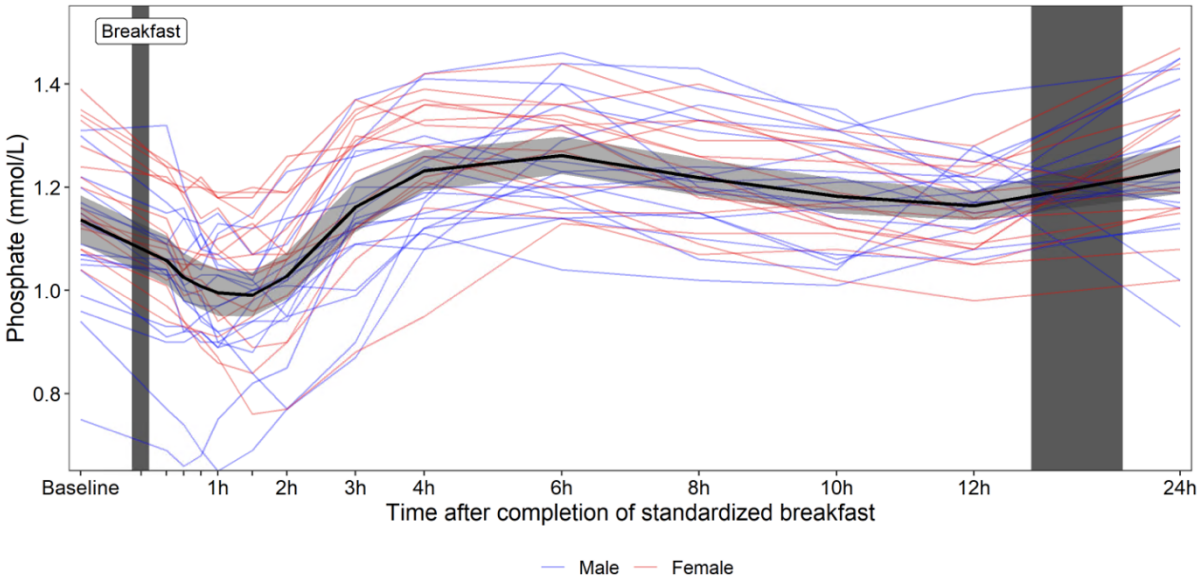

Figure 4.4: ***Quantified serum phosphate (mmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

4.3.2 Lipids and transport molecules

Triglycerides

Figure 4.5 expresses the fluctuations of serum triglycerides postprandially and in transition to the fasting state. Initially, there was an increase in the triglyceride's circulating concentrations, which peaked nearly 4 hours postprandially (gMean 1.10 mmol/L). Then the circulating concentration of the biomarker decreased, and around 6 hours after completion of the breakfast meal, the circulating concentrations were back to baseline levels (see Table 4.2). The minimum concentration of the biomarker was reached approximately 8 hours after the initial time of intervention (gMean 0.72 mmol/L). Then the biomarker stabilised until it increased again 12 hours after completing the breakfast meal. When participants returned for their last blood sample on the following day, the circulating concentrations were back to baseline levels. On average, there was approximately a 0.38 mmol/L difference between the maximum and minimum concentrations during the study period. By studying the intraindividual variation of the biomarker, there were observed some fluctuations of the biomarker for the first 3 hours postprandially. However, the circulating concentrations primarily followed the same pattern as the gMean line.

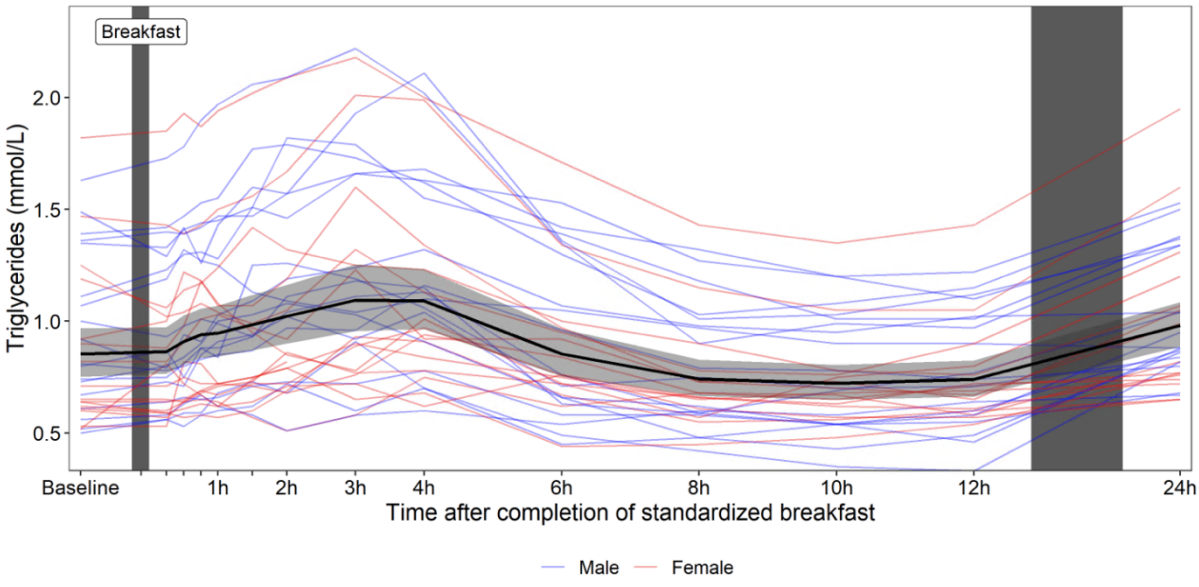

Figure 4.5: ***Quantified serum triglycerides (mmol/L) as a function of time since completion of the breakfast meal****. Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

Low-density Lipoprotein

The measured concentrations of LDL plotted as a function of time are displayed in Figure 4.6. Overall, the biomarker was relatively stable from baseline and throughout the intervention, with virtually no notable variations in the circulating concentrations. However, within the first to hours after completion of the standardised breakfast meal, there were small fluctuations in the biomarker at an intraindividual scale. The gMean baseline concentrations started at 2.51 mmol/L and ended at a maximum of 2.68 mmol/L. Male participants had, on average, the highest-circulating concentrations, with baseline concentrations of gMean 2.58 mmol/L vs. female participants gMean 2.44 mmol/L.

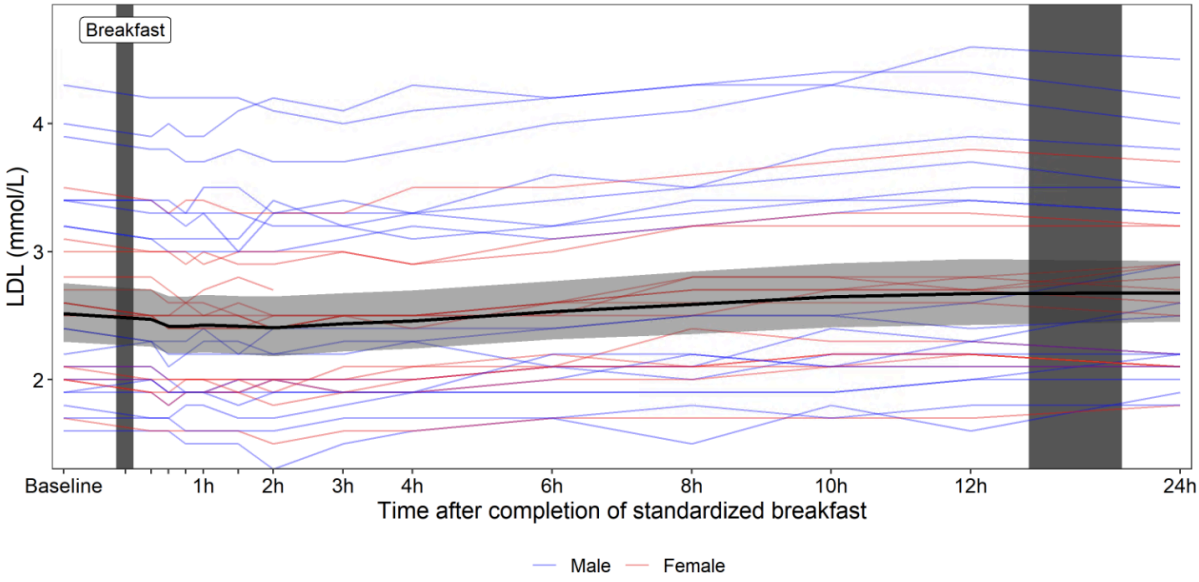

Figure 4.6: ***Quantified serum low-density lipoprotein (LDL) (mmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

High-density lipoprotein

Figure 4.7 graphically presents the measured concentrations of HDL. Similar to the LDL levels, HDL concentrations were relatively stable from baseline (gMean 1.55 mmol/L) and throughout the postprandial- and fasting state. There was a slight increase in the circulating concentrations from the meal consumption to the 12th hour initially, followed by a slight decrease in circulating concentrations during the night. On average, female participants had higher HDL concentrations than male participants (Table 4.2). For the first two hours after completion of the standardised breakfast meal, the HDL concentrations fluctuated slightly at an intraindividual level.

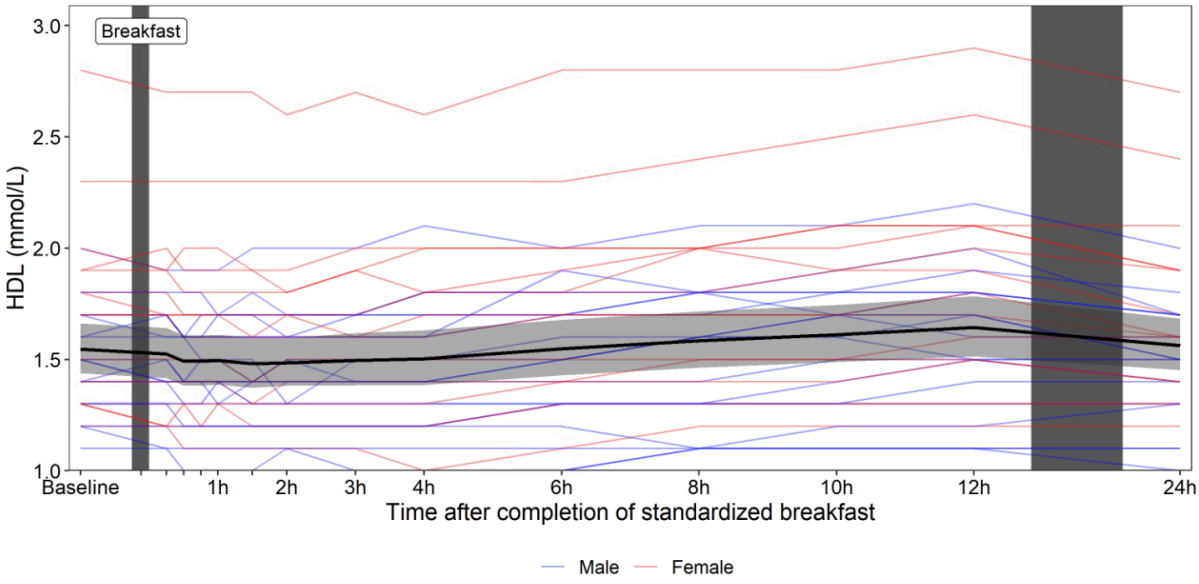

Figure 4.7: ***Quantified serum high-density lipoprotein (HDL) (mmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

Albumin

Time course presentation of the measured albumin concentrations is displayed in Figure 4.8. Overall, albumin was relatively stable from baseline and throughout the intervention. There was a slight increase in the biomarker from the consumption of the breakfast meal (gMean 45.2 g/L) until 12 hours postprandially (gMean 48.0 g/L). Then there was a slight decrease in the biomarker over the night. At the last blood sampling, the circulating concentration of the biomarker was slightly above the baseline concentrations (gMean 47.4 g/L). The baseline concentrations range from 40 g/L to 49 g/L, where the male participants have the highest values (Table 4.2). At the intraindividual level, the biomarker fluctuated some until 2 hours postprandially.

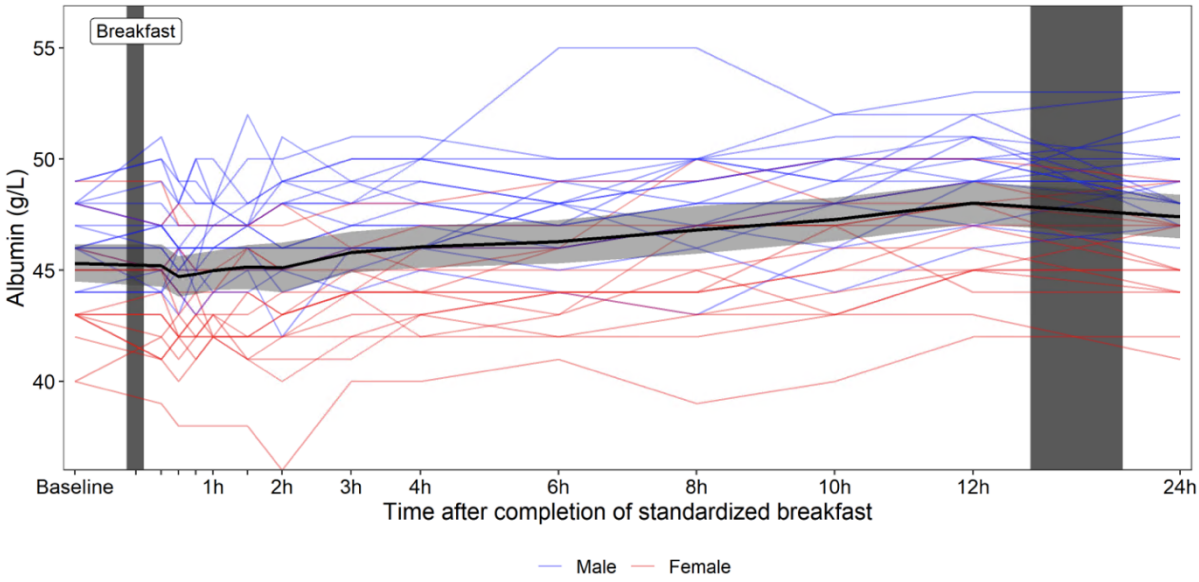

Figure 4.8: ***Quantified serum albumin (g/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

4.3.3 Vitamins

Folate

Figure 4.9 graphically presents the measured concentrations of folate. During the first hour of the intervention, some fluctuations in the intraindividual measurements were observed. The graph also illustrates that there were some interindividual variations in circulating concentrations. The gMean concentration started at 13.8 nmol/L, and at 24 h after the breakfast, the gMean concentration increased to 22.0 nmol/L for the last blood sampling, a difference of 8.2 nmol/L. The overall trend in the circulating concentration of the biomarker was a slight increase from baseline to the end of the intervention.

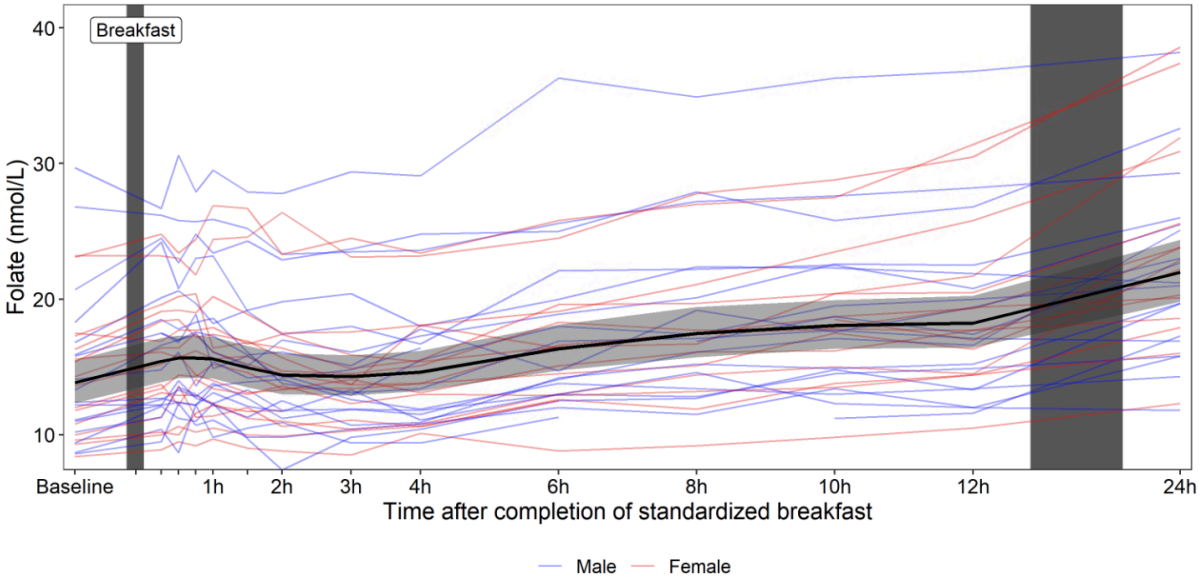

Figure 4.9: ***Quantified serum folate (nmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre. Due to hemolysis, four measurements have been excluded from the individual timeline; two of the excluded measurements are within the same male participant (the 1st and 10th time sample) and the 11th time point in another male and female participant.*

Cobalamin

Time course presentation of the measured cobalamin concentrations is displayed in Figure 4.10. During the first two hours after consumption of the breakfast, there were some fluctuations of the biomarker at an intraindividual scale. However, looking at the gMean concentration, the timeline indicates no fluctuations within this time interval. Overall, there was a slight increase in circulating concentrations of cobalamin from the consumption of the breakfast until 24 hours after the breakfast, where gMean concentration started at 328 pmol/L and increased to 379 pmol/L, a difference of 51 pmol/L. The figure also illustrates some interindividual variation in circulating concentrations, with baseline concentrations ranging from 176 pmol/L to 675 pmol/L.

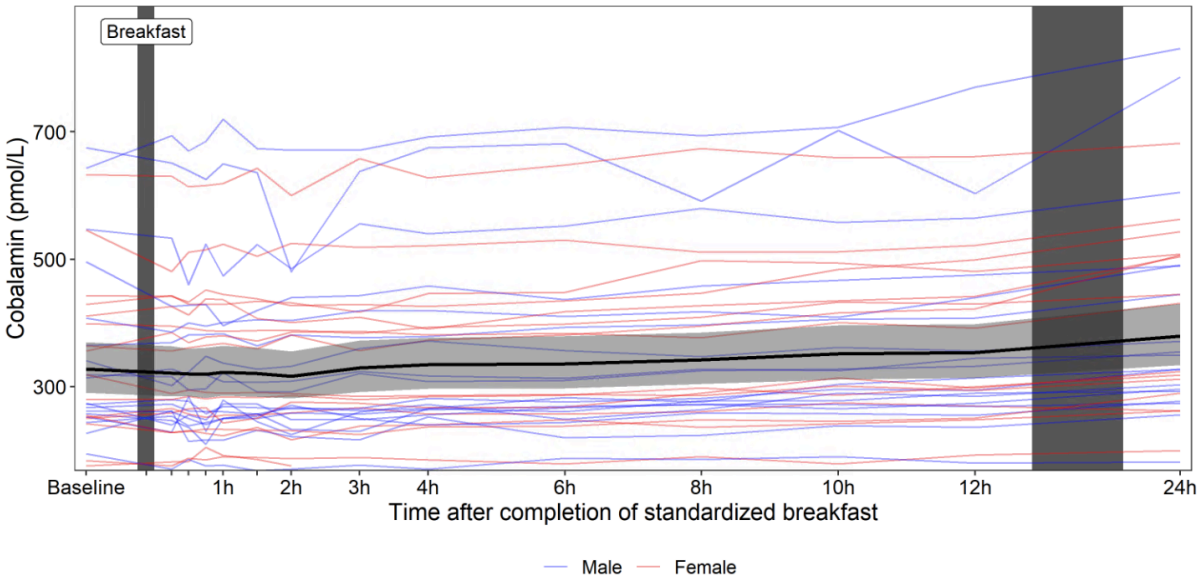

Figure 4.10: ***Quantified serum cobalamin (pmol/L) as a function of time since completion of the breakfast meal.*** *Blue lines represent males, while the red lines represent females. The solid black line represents the geometric mean concentration for the whole study population, and the shaded area around this line illustrates the 95% geometric confidence interval. The leftmost vertical line indicates the time of breakfast and the initial time of intervention. The broader line to the right indicates time spent outside the study centre.*

4.4 Intraclass correlation coefficients of biomarkers

ICCs for the biomarkers, calculated based on absolute agreement across time points, are presented in Table 4.3. For the whole study population, ICCs ranged from 0.37 – 0.82. Phosphate (ICC = 0.37) was the only biomarker with a score under 0.50, conventionally interpreted as indicating poor reliability. All biomarkers except HDL (ICC = 0.82) and triglycerides (ICC = 0.76) had an ICC between 0.50 ≤ and < 0.75, indicating moderate reliability. Male participants had higher ICCs for all biomarkers except phosphate (0.28 vs 0.52) compared to female participants. In the female study population, triglycerides were the biomarker with the highest ICC, with an ICC of 0.78.

Table 4.3: Calculated intraclass correlation coefficients of the biomarkers in PoMet-study.

Biomarker	Total population (n = 34)	Males (n = 18)	Females (n = 16)
Potassium	0.50	0.51	0.50
Phosphate	0.37	0.28	0.52
Magnesium	0.64	0.75	0.57
Triglycerides	0.76	0.75	0.78
High density lipoprotein	0.82	0.94	0.71
Low density lipoprotein	0.68	0.97	0.57
Albumin	0.50	0.55	0.50
Folate	0.53	0.63	0.51
Cobalamin	0.51	0.97	0.51

5.0 Discussion

For interpreting the results of biomarkers in blood samples, especially nutritional biomarkers, prandial status can be challenging. The continuous access to food, particularly in the western part of the world, causes us to be in the postprandial state for most of the day. We usually don't enter the postabsorptive state before falling asleep and after an overnight fast. This results in blood samples often being taken in the postprandial state unless patients (or study subjects) have early doctor's visits. The time-resolved data provided on a range of biomarkers during the postprandial period from this study allows us to identify the biological changes of these biomarkers after a meal. The present study contributes to knowledge of the impact of the timing of the last meal, which is beneficial when interpreting blood samples in the clinic, as well as in research for biomarker-health associations, where the information can be used to reduce the impact of intraindividual variation in these biomarkers. As the study population consists of males and females, sex-specific differences in the biomarkers are also accentuated in this thesis. First, some methodological considerations need to be discussed.

5.1 Methodological considerations

5.1.1 Inclusion and exclusion criteria

The inclusion and exclusion criteria for participating in the study were relatively narrow and specific; All participating study subjects were young, healthy, and had a BMI within the normal range. These criteria were set to secure the accomplishment of collecting data from a homogenous population, which increases the study's internal validity. On the other side, these requirements may limit the generalisability of the findings to the general population, consisting of all ages with varying health statuses.

Unfortunately, a study including fasting for 24 hours could attract people struggling with food resulting in a selection bias which would reduce the generalisability of the general population. The self-reported BMI criteria of 22.0-27.0 kg/m^2 were given to mitigate this potential bias and secure a healthy study population.

Some of the recruited participants were below the "self-reported"- BMI criteria and should ideally have been excluded from the study. However, the wording "self-reported" is a conscious choice as it might be a bias between scales at home versus the study centre, clothing and

hydration status. There was also a possibility that the study subjects either over-or underestimated their height prior to screening. The measurement of height is also dependent on the timing of the day for measurement. Because the BMI criteria were set to secure recruitment of healthy individuals and limit large variations within BMI, some participants were included even though they had a BMI below 22.0 kg/m^2 at the study visit.

When it comes to the inclusion of female participants, first, we wanted to delimitate the recruitment of those not using any contraceptives. The argument was that different contraceptives impinge, in varying degrees, on the body's hormone systems and the metabolome (44-46). The delimitation made it difficult to recruit female participants, and the decision was made to remove this criterion. Nevertheless, this decision might decrease the internal validity but, oppositely, increase the female population's external validity and thereby the generalisability.

Overall, the study's final inclusion and exclusion criteria have emphasised internal validity over external validity, as recommended by Rothman et al. when investigating causal relationships (67). However, to extend our findings, future studies should consider postprandial and postabsorptive metabolism in other populations with regard to age, body composition, and health status.

5.1.2 Preparation of study subjects prior to the study visit

One week prior to the study visit, the participants were instructed not to use any dietary supplements, and in the last 24 hours before the study visit, abstain from alcohol and avoid strenuous physical activity. These requests standardise the physiological and metabolic interindividual differences in the participants' metabolome. Interindividual differences in the baseline anthropometric measurements and blood sample at attendance were additionally normalised by instructing the participants to eat a meal similar to the standardised breakfast meal (Table 3.2) at 8 p.m. the evening before the study visit (26). All these requests increase the study's internal validity, contributing to a more homogenous study population and maximising comparability between subjects.

The request to eat an evening meal similar to the standardised breakfast meal also allows the possibility of comparing the overnight fast (in baseline samples) with the fasting during the

day (samples collected 12 hours after completion of the standardised breakfast meal). This information assesses the possibility of investigating circadian rhythm alterations' impact on the biomarkers of interest. After collecting the information on how the participants had prepared for the study visit, most participants had eaten the requested meal the previous evening at 8 p.m. Therefore, we can conclude that we accomplished normalising potential interindividual differences in the metabolome.

A few participants had another meal the previous evening at 8 p.m. This has not been emphasised when interpreting the results since it is assumed it will have a limited impact on the overall results of the intervention. However, if we had served the evening meal before the study visit, this situation could have been avoided. On the other hand, serving the evening meal would have caused a more considerable burden on the participants, requiring them to show up an extra day.

5.1.3 The breakfast meal

We chose to serve the study subjects a standardised breakfast meal to describe biomarkers' biological movement during the postprandial state. This meal was supposed to mimic an average Norwegian diet regarding foods and macronutrients, imitating a "real-world" context.

However, designing the study this way poses some challenges, as there previously has been illustrated a considerable interindividual variation in the postprandial metabolic response to identical meals (68). Consequently, to standardise physiological and metabolic conditions, which influence the circulating concentrations of metabolites, the participants were served the same standardised breakfast meal, which they consumed within the same time (15 minutes). This limits substantial noise in the measurements caused by eating speed on digestion and the release of nutrients into circulation. It will also maximise interindividual comparability and increases the study’s internal validity.

All the participants in our study consumed the same standardised breakfast meal at the same pace and time of day. It is essential to emphasise that because all participants ate the same standardised breakfast meal, at the same time of day and at the same pace, the results can not necessarily be extrapolated to meals with different compositions or eaten at other times. This should be addressed in future studies. Furthermore, all participants were provided with the same size meal in terms of caloric content. In line with the recommendations, the breakfast

meal should provide 20-30% of the daily energy intake. As the resting metabolic rate differ substantially between participants, the relative contribution to the total daily energy requirement differs, which might have influenced the results.

5.1.4 Collection of data

All the collected anthropometric measurements and blood samples in the study were collected and handled by the same trained personnel, following standard operating procedures (Appendix 2), which drastically reduces differences in sample collection and handling of blood samples after collection. This also increases the quality of the collected samples by reducing the possibilities for subjective variations in the anthropometric measurements and limiting the preanalytical variations regarding the collection and handling of blood samples, such as individual perception of time.

The intervals of blood sample collection were based on the assumption of increased fluctuations of the biomarkers in the first hours of the postprandial state with a decrease as the closer the postabsorptive state gets, and explains the distribution of the 14-time points of blood sampling in the study. The frequent sampling during the postprandial period within each participant is quite unique and gives comprehensive information on metabolite concentrations during the adaption to the fasting state. The standardisation of the timing of data collection events further reduces the interindividual variations in the collected data. Finally, it should also be mentioned that we, with a few exceptions due to the occlusion of the venous catheter and for the 24-hour sample, managed to draw all blood samples within ± 2 minutes from specified points in time. The 24-hour sample was drawn the following morning at a convenient time with respect to participants' job and school agreements and may thus deviate somewhat from the specified time.

5.1.5 Analytical considerations

All measured biomarkers of interest were quantified at the department of Medical Biochemistry and Pharmacology at Haukeland University Hospital Bergen, Norway, which is accredited by NS-EN ISO 15189:2012. The NS-EN ISO 15189:2012 accreditation is a high-quality sign as it demands strict requirements of quality assurance systems for monitoring and documenting the quality of analyses.

The biomarkers of interest are reported to be stable for five days if stored in the fridge or at room temperature. The aliquoted serum was delivered to MBF on the day of intervention completion and analysed within the following 24 hours, which is well within the stated durability limits for the analyses. All analyses of biomarkers of interest were also quantified using the same instrument, eliminating the between-instrument variation.

5.2 Discussion of results

5.2.1 Study subjects

The aim of collecting complete data from 32 study subjects was reached, with 33 participants completing the intervention. Based on the baseline characteristics reported in Table 4.1, we succeeded in collecting a relatively homogenous sample on most parameters in accordance with the inclusion and exclusion criteria.

The formula of BMI only considers weight and height and don't consider body composition. It can, therefore, in some cases, over-and underestimate adiposity. The distribution of baseline characteristics (Table 4.1) further illustrates that our study population had various body compositions. The fat percentage is quite diverse within the population, even if the calculated BMI is considered within the normal range. The inverse correlation between body composition in terms of total body fat and insulin sensitivity, discussed in section 1.1.1, further indicates that the variation in fat percentage might cause a variation in insulin sensitivity between subjects (16, 17). In context with the known impact insulin has on, for instance, circulating concentrations of phosphate (10, 33) and potassium (32), can the variation in fat percentage between subjects cause a variation in the circulating concentrations of biomarkers influenced by insulin. This potential variation could have been avoided if we had recruited subjects according to fat percentage instead of BMI. However, it would have made the recruitment process more expensive and demanding regard to time. In addition, it would have caused a more considerable burden on the participants, requiring them to show up an extra day or show up and then be excluded from participating in the study.

5.2.2 Time-resolved changes in postprandial biomarker concentrations

This section will discuss possible causes of the varying fluctuations observed in the investigated biomarkers (Figure 4.2-4.10) and whether there is a causality between the time of last meal and the observed fluctuations in the biomarkers.

Postprandial response in the first 4 hours of intervention

First, the varying observed interindividual variation in fluctuations, particularly for the first 4 hours of intervention in all the biomarkers presented in Figures 4.2-4.10, might be explained by interindividual variation in the postprandial metabolic response. Yet, it should also be mentioned there is an underlying influence of circadian rhythms in the time-resolved changes in postprandial biomarker concentrations, which will be further discussed in the following sub-section. When studying the respective graphs, the fluctuations of the biomarkers potassium, phosphate and triglycerides follow distinct patterns. Based on previous studies on postprandial metabolism of these biomarkers discussed in section 1.4 in the introduction, the pattern of the concentration changes in the first 4 hours of intervention was expected can be explained.

Potassium

For potassium (Figure 4.2), the observed decrease in circulating concentrations in the first hour is presumably due to the intracellular shift in the $Na^{+/}K^{+}$ ATPase induced by insulin (32). The following increase in circulating concentrations for the next three hours can be explained by the absorption of dietary potassium from the GI-tractus. After the 4^{th} hour, postprandially, the point of inflection in the curve indicates the end of dietary absorption and the excretion effect of excessive potassium in the kidneys appears.

Phosphate

In the first hour postprandially of phosphate (Figure 4.4), the decrease in circulating concentrations is most likely explained by insulin stimulating cellular uptake of phosphate (10, 33). The absorption of dietary phosphorus can explain the following increase in the circulating concentrations with a simultaneous decrease in insulin excretion from the pancreas as the postprandial period diminishes and gradually passes over into the postabsorptive state.

Triglycerides

For triglycerides (Figure 4.5), the rise in the circulating concentrations after completion of the standardised breakfast meal, which peaks between 3-4 hours postprandially, is presumably a result of the absorption of dietary triglycerides from the intestine (3, 35). The following inflection point in the triglycerides curve indicates the end of dietary absorption and the metabolism gradually passing over into the postabsorptive state.

Magnesium, HDL, LDL, albumin, folate, and cobalamin

For magnesium (Figure 4.3), HDL, LDL, albumin, folate, and cobalamin (Figures 4.6-4.10), the interindividual fluctuations during the first 4 hours do not follow a distinct pattern. This explains why the gMean timeline indicates virtually no or minor changes in the circulating concentrations during the first 4 hours of intervention.

The analytical variation of all these biomarkers, except the vitamins folate and cobalamin, is considerably low, varying from 2.5-3.0% (Table 3.3). Taking into consideration that the intraindividual measurements have been analysed within the same batch further limits the analytical variation of these biomarkers. Analysing the samples in one set eliminates potential variations originating from LOT-to-LOT, reagent to reagent, and possible analytical variations from the reagent's lastingness. Furthermore, the preanalytical influence on measures of these biomarkers is also limited because of the standardisation of sample collection and handling. Thus, the observed fluctuations in these biomarkers seem to be random and occur due to minor interindividual differences rather than a systematic outcome from the breakfast meal.

The analytical variation of folate (10%) and cobalamin (7%) are appreciable higher than for the other biomarkers. Thus, we cannot conclude whether the observed changes in folate and cobalamin alone result from the meal, interindividual differences or analytic variations or whether these observations are a combination of them all. Finally, it should be noted that the potential influence from an analytical variation should be “random error” and not appear as a systematic pattern over several time point as illustrated in these biomarkers.

The transition from the postprandial to the postabsorptive state

First, fasting increases the risk of dehydration, and the possibilities of different individual metabolic responses to dehydration might cause the observed interindividual variations in all biomarkers during this time of intervention (19).

Potassium, phosphorus, triglycerides and albumin

For all biomarkers of interest, except folate and cobalamin, the overall trend is fewer fluctuations in the metabolite concentrations after completion of the first 4 hours of intervention. However, the continuous decrease in circulating concentrations of potassium, phosphorus and triglycerides, and oppositely an increase in albumin concentrations until the 12th hour after completion of the standardised breakfast meal raises an interesting question. These changes might be explained by the homeostatic regulation of the metabolites as the circulating concentrations reflect vital processes in the metabolome. Such as absorption and release of molecules from cells, tissues and excretion, absorption and reabsorption occurring in, for instance, kidneys, liver and lungs.

Optionally, these changes might occur as a result of underlying variations caused by circadian rhythms, which is further substantiated by the baseline concentration being near the same concentrations as the 24-hour sample. It should be mentioned that it's known that the circulating concentrations of phosphorus increase during the night (10). However, it has previously been suggested that the rise in circulating concentration associated with circadian rhythm changes on a large scale occurs as a secondary effect of food intake (34). Thus, the continuous observed increase in circulating phosphorus during the night occurs due to not eating after 8 p.m. This agrees with our study's pattern with the highest circulating phosphorus concentrations 2-10 hours postprandially. Still, these assumptions are just speculation as the effect of circadian rhythms on nutritional biomarkers is challenging to quantify since it requires blood samples after standardised meals at all hours of the day to isolate the factor of circadian rhythms.

Folate and cobalamin

For the vitamins folate and cobalamin, the circulating concentrations constantly increase from around the 3-4 hours postprandially and throughout the intervention. The observed increase of these two biomarkers cannot be explained by the analytical variation alone, indicating the involvement of other mechanisms. There has previously been illustrated a correlation between

fasting and an increase in circulating folate levels (50). A simultaneous rise in cobalamin concentrations, at the same time in intervention, causes some curiosity. Both vitamins are linked together in several processes in the one-carbon metabolism, and the possible answer to the continuous increase in the circulating concentrations of these two biomarkers might lay there. A previous study has observed increased remethylation of homocysteine to methionine when the ingestion of preformed methyl moieties was curtailed (69). A process which is dependent on both folate and cobalamin. If we put this in context with the observations from our study, the simultaneous rise in cobalamin and folate further into the fasting state could be explained by a mobilising of the vitamins because of increased requirements. However, further research must be done to conclude whether the observed increase in the circulating concentrations is connected.

Magnesium, HDL, LDL

There are no notable changes in the metabolite concentrations except for some individual exceptions in this part of the intervention in magnesium, LDL and HDL.

5.2.3 Sex-specific differences in the baseline data and the time-resolved presentation

There are minor differences in the baseline concentrations between the sexes (Table 4.2). However, for albumin, female participants had a gMean concentration of 43.9 g/L compared to gMean male concentrations of 46.6 g/L. The distinct difference in the albumin concentration is further illustrated in the time-resolved presentation of the metabolite. The difference might be explained by the fact that contraceptives lead to lower circulating albumin concentrations, as described in section 1.4. All the female participants, except two, used different contraceptive types. The remaining two female participants without contraceptives were in the same concentration range as the male participants. This substantiates the assumption that contraception also influences the circulating concentrations of other biomarkers. Therefore, the time-resolved presentation of biomarkers in female participants is probably, to some extent, influenced by contraceptives. It should also be mentioned that the female participants also had their study visits at different times of their menstrual cycle, which might have caused variations between all the female participants as the hormones also vary at different stages of the cycle (30). However, further research must be done before concluding which other and how

biomarkers are affected by these hormonal differences caused by contraception and the menstrual cycle.

5.2.4 Intraclass correlation coefficients of biomarkers

ICC was used to evaluate the absolute agreement in biomarker concentrations between the 14 samples of each individual and quantifies the interindividual and intraindividual variations. In this study, where we have studied the fluctuations in the context of time since food intake, the calculated ICCs can be used to decide whether the time of last meal is an essential factor for the intraindividual variance. The result can further be used to address whether the measurement of a single sample at a random time since the last meal is representative of the person's metabolite concentrations over the entire 24 h period.

Collectively, looking at the whole study population (Table 4.3), phosphate had poor reliabilities suggesting a significant within-person variance in the postprandial/postabsorptive period. When evaluating this biomarker, the time of the last meal appears essential. All other metabolites had moderate reliabilities except triglycerides and HDL, suggesting a sizeable within-person variance. The time of last meal is still an important factor when evaluating these biomarkers. Triglycerides and HDL had good reliabilities in the calculated ICCs, indicating that the between-person variance explains most of the total variance, with less within-person variability. When evaluating triglycerides and HDL measurements, the last meal's time appears to be of less importance.

In general, the ICCs were lower in females compared to males. This suggests that the within-person variation was more considerable in women and that standardising the blood sampling time point relative to food intake may be of more importance in females. A potential explanation for this may be the additional variability introduced by the differences in contraceptive use and menstrual cycle. Regardless, it must be mentioned that males' ICCs are also considerably low for most metabolites. Thus, we can conclude that time point relative to food intake is vital for both sexes, with some exceptions.

5.2.5 Implications of this study

Based on the findings from this study, it is natural to re-address the dilemma discussed in section 1.3 of the conventional, binary classification of blood samples into non-fasting and

fasting samples. The time-resolved changes in postprandial biomarker concentrations illustrate fluctuations in metabolite concentrations, particularly during the postprandial state in many of the investigated biomarkers. Furthermore, the presentation of time-resolved changes in postprandial biomarker concentrations demonstrates that the metabolite concentrations change dynamically, with no sharp discontinuities where a natural cutpoint could distinguish between fasting and non-fasting.

The collected information in the present study will have implications for the clinic and research. In the clinic, knowledge regarding the time of the last meal will allow for the possibility of taking prandial status into account when interpreting the results. Further, the possibility to estimate, for instance, 12-hour fasting concentrations would facilitate easier comparability between samples collected at different postprandial time points. This allows the opportunity to estimate, for example, 12-hour fasting concentrations. Standardising the time point of blood samples after a meal will also be valuable in dietary studies, when it is necessary to know the timing of the last meal precisely and when the binary classification of fasting and non-fasting blood samples is not suitable. In nutritional research, particularly epidemiological studies, investigating the association between biomarkers and disease demands a higher requirement for standardising blood samples' timing regarding the timing of the last meal. If the blood samples were taken at different times after the last meal, this would introduce unwanted interindividual variation in the biomarker concentrations. In that case, it is essential to assess how much variation in the biomarkers this may have contributed. This information may be used to adjust the statistical models or to standardise the concentrations by estimating the concentrations at a specific time point. Finally, the collected data indicates when the last meal's time point is less important, which can be helpful in the clinic where standardising prandial status can be challenging.

6.0 Concluding remarks

When interpreting the measured concentrations of nutrition-related biomarkers, time since the last meal is a factor which could be of importance. This study observed considerable fluctuations, particularly in the early postprandial state, for several metabolites such as electrolytes and triglycerides. A steady increase throughout the postprandial and postabsorptive state was observed for folate and cobalamin. However, time since the last meal is not necessarily equally critical for all metabolites. In this study, the magnesium, LDL, HDL, and

albumin concentrations were relatively stable during the intervention period. Overall, the intraindividual fluctuations were larger in females compared to males.

This study increases our understanding of the postprandial changes in metabolite concentrations, which may have extensive implications both in the clinic and in research. To complement our findings, future research should examine the postprandial and postabsorptive metabolism in different populations and after meals with different compositions and eaten at other times of the day.

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Appendices:

Appendix 1: Case record form

Appendix 2: Standard operating procedures

Appendix 1

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)

The PoMet-study

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

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					
Title	Screening by phone	Page	2	Of	3
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					
Title	Screening by phone	Page	3	Of	3
Version	1	Created on		25.06.2021	
Status	Draft				
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.

Standard operating procedure					
Title	Study visit	Page	2	Of	5
Version	1	Created on		09.07.2021	
Status	Draft				
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					
Title	Study visit	Page	3	Of	5
Version	1	Created on		09.07.2021	
Status	Draft				
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					
Title	Study visit	Page	4	Of	5
Version	1	Created on		09.07.2021	
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.

Standard operating procedure					
Title	Study visit	Page	5	Of	5
Version	1	Created on		09.07.2021	
Status	Draft				
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

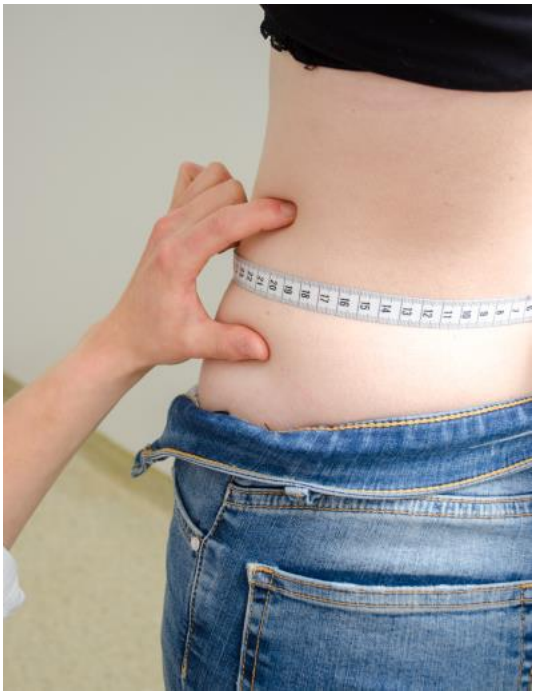

Figure 1 - Correct placement of the measurement tape

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