The dynamics of nutritional-related blood biomarker and metabolite concentrations during the postprandial and fasting states

And the implications when using nutritional-related biomarkers and metabolites in clinical care and research settings

Åslaug Matre Anfinsen

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The project culminating in this thesis was carried out from July 2020 to December 2023 at the Centre for Nutrition, Department of Clinical Science, Faculty of Medicine, University of Bergen. My main supervisor has been Dr. Vegard Lysne, and my co-supervisors have been Dr. Hanne Rosendahl-Riise, Dr. Adrian McCann, and Professor Ottar Kjell Nygård.

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Abbreviations

Acetyl-CoA	Acetyl coenzyme A
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acids
βHB	β-hydroxybutyrate
BMI	Body mass index
CV	Coefficient of variation
CETP	Cholesteryl ester transfer protein
EDTA	Ethylenediaminetetraacetic acid
FMN	Flavin mononucleotide
GC-MS/MS	Gas chromatography-tandem mass spectrometry
gCI	Geometric confidence interval
gMean	Geometric mean
gSD	Geometric standard deviation
gSE	Geometric standard error
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HUSK	Hordaland Health Study
ICC	Intraclass correlation coefficient
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MMA	Methylmalonic acid
mNAM	N1′-methylnicotinamide

PI	Prediction interval
PLP	Pyridoxal-5´-phosphate
PoMet study	Postprandial Metabolism study
REK	Regional Committee for Medical and Health Research Ethics
SD	Standard deviation
TCA	Tricarboxylic acid
TMP	Thiamine monophosphate
VLDL	Very-low-density lipoprotein

Summary

BACKGROUND: Little is known about the dynamics of nutritional-related blood biomarkers and metabolite concentrations during the postprandial and fasting states.

OBJECTIVE: To explore the dynamics of blood concentrations of amino acids, one-carbon metabolites, vitamins, lipids, ketones, and acylcarnitines during 24 hours following dietary intake.

METHODS: In **Paper I**, we used cross-sectional data from the HUSK study, and included 2960 middle-aged and 2874 elderly adults. Blood samples were collected once from each participant, and the number of hours was categorized from 0 to 7 hours after a meal. Marginal gMean (95% gCIs) concentrations of amino acids, one-carbon metabolites, lipids, and markers of vitamin status were estimated from a linear regression model adjusted for sex, age group, and BMI and presented as a function of time since the last meal. **Papers II** and **III** were based on the interventional PoMet study, which included 34 participants aged 20-30 years. Participants were served a standardized breakfast meal and consumed only water for the next 24 hours. Blood samples were taken at baseline and at 13 standardized time points from 15 minutes to 24 hours after the meal. gMean (95% gCI) concentrations of amino acids, one-carbon metabolites, and B-vitamin biomarkers (**Paper II)** and lipids, ketones, and acylcarnitines **(Paper III**) were plotted as a function of time since the breakfast meal.

RESULTS: Considerable changes in concentrations were found for nearly all amino acids and one-carbon metabolites, several vitamin biomarkers including thiamine, TMP, FMN, cobalamin, folate, and phylloquinone, ketones, free carnitine, and short- and medium-chain acylcarnitines. Modest changes were also found for triglycerides, LDL- and HDL cholesterol in the hours after dietary intake.

CONCLUSION AND IMPLICATIONS: Our findings suggest that it is not sufficient to merely distinguish between non-fasting and fasting blood samples when using nutritional-related biomarkers and metabolites in clinical settings and epidemiological studies. Accounting for prandial status should be done by evaluating the exact time since the last meal.

Sammendrag

BAKGRUNN: Vi vet lite om hvordan ernæringsrelaterte biomarkører og metabolitter endrer seg i blodet i postprandiell og fastende tilstand.

MÅL: Å undersøke hvordan konsentrasjonen av aminosyrer, en-karbon metabolitter, biomarkerører for vitaminstatus, lipider, ketoner, og acylkarnitiner endrer seg i timene etter matinntak, med fokus på de første 24 timene.

METODE: I **Artikkel I** brukte vi tverrsnittsdata fra HUSK-studien, og inkluderte 2960 middelaldrende og 2874 eldre voksne. Blodprøver ble tatt en gang fra hver deltaker, og tid siden siste måltid ble kategorisert i timeskategorier fra 0-7 timer. Marginale geometriske gjennomsnitt (95% geometrisk konfidensintervall) av konsentrasjonen av aminosyrer, en-karbon metabolitter, lipider, og biomarkører for vitaminstatus ble estimert fra en lineær regresjonsmodell justert for aldersgruppe, kjønn og kroppsmasseindeks, og presentert som en funksjon av tid siden siste måltid. **Artikkel II** og **III** var basert på intervensjonsstudien PoMet, som inkluderte 34 deltakere i alderen 20-30 år. Deltakerne spiste en standardisert frokost og inntok deretter kun vann i de neste 24 timene. Blodprøver ble tatt ved baseline og på 13 standardiserte tidspunkt fra 15 minutter til 24 timer etter måltidet. Geometrisk gjennomsnittlig (95% konfidensintervall) konsentrasjon av aminosyrer, en-karbon metabolitter, og B-vitamin biomarkører (**Artikkel II**), og lipider, ketoner, og acylkarnitiner (**Artikkel III**) ble plottet som en funksjon av tid siden frokostmåltidet.

RESULTAT: Betydelige endringer i konsentrasjon ble observert for nesten alle aminosyrer, en-karbon metabolitter, flere biomarkører for vitaminstatus, inkludert tiamin, TMP, FMN, kobalamin, folat og fyllokinon, ketonene, fritt karnitin, og de korte- og middelkjedete acylkarnitinene. Det ble også funnet moderate endringer i konsentrasjonen av triglyserider, LDL- og HDL kolesterol i timene etter matinntak.

KONKLUSJON: Våre funn tyder på at det ikke er tilstrekkelig å bare skille mellom ikke-fastende og fastende blodprøver når man bruker ernæringsrelaterte biomarkører og metabolitter i klinikk og i forskningssammenheng. Man bør ta høyde for prandiell status ved å ta hensyn til den nøyaktige tiden siden siste måltid ved blodprøvetaking.

List of publications

I. Anfinsen ÅM; Rosendahl-Riise H; Nygård O; Tell GS; Ueland PM; Ulvik A; McCann A; Dierkes J; Lysne V (2023): *"Exploratory analyses on the effect of time since last meal on concentrations of amino acids, lipids, one-carbon metabolites, and vitamins in the Hordaland Health Study."* European Journal of Nutrition. Doi: 10.1007/s00394-023-03211-y

II. Anfinsen ÅM; Johannesen CO; Myklebust VH; Rosendahl-Riise H; McCann A; Nygård O; Dierkes J; Lysne V (2023): "*Time-Resolved Concentrations of Serum Amino Acids, One-carbon Metabolites, and B-vitamin Biomarkers during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study*." British Journal of Nutrition. Doi: 10.1017/S0007114523002490

III. Anfinsen, ÅM; Myklebust VH; Johannesen CO; Christensen JJ; Laupsa-Borge J; Dierkes J; Nygård O; McCann A; Rosendahl-Riise H; Lysne V: *"Serum concentrations of lipids, ketones, and acylcarnitines during the postprandial and fasting states: The Postprandial Metabolism in Healthy Young Adults (PoMet) study."* Manuscript submitted December 2023.

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1. Introduction

Today, thousands of metabolites in humans are identified, with many utilized as biomarkers. Biomarkers, short for biological markers, have been used in clinical care for decades, serving various critical purposes, including assessing health status, gauging disease, and monitoring treatment responses. Biomarkers and metabolites are also used extensively in both interventional research and observational epidemiological studies. However, the concentrations of biomarkers and metabolites in the blood may vary during the day, and changes in energy metabolism are one of several factors that influence biomarker and metabolite concentrations. In this section, an introduction to the field of biomarkers and metabolites will be given, followed by a description of human energy metabolism.

1.1 Biomarkers and metabolites

The term metabolome was first used by Oliver et al. in 1998 (1) and can be defined as the quantitative complement of all the low-molecular-weight molecules, known as *metabolites*, present in cells in a particular physiological or developmental state (2). As of 2022, a total of 217,920 metabolites were identified in the Human Metabolome Database (3), and the number continues to increase. Metabolites may provide crucial information about an underlying biological state, and thus, research investigating the use of metabolites as biomarkers to assess health and disease status and predict future health outcomes has grown exponentially over the last three decades, a trend that is expected to continue (4, 5).

The definition of a “biomarker” has been subject to considerable ambiguity, with multiple interpretations prevailing. This issue became apparent at a joint leadership conference of the U.S. Food and Drug Administration and the National Institutes of Health in 2015, where it became evident that the leaders of these federal agencies had different conceptions of the appropriate definitions of biomarkers in different contexts of use (6). Consequently, recognizing the need for a standardized

definition, a dedicated task force was established to provide a unified understanding of biomarkers through the "Biomarkers, Endpoints, and Other Tools" (BEST) resource. The first version of the glossary in the BEST resource was published in 2016 and defines a biomarker as *"a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention"* (7). Importantly, in contrast to medical symptoms, which are perceived by the patients themselves, biomarkers are objective measurements and do not necessarily correspond to the individual's own experience of health and well-being (8).

Biomarkers may be broadly categorized into three groups: Physical or genetic traits (such as blood pressure or metabolic gene polymorphisms), chemical or biological agents in the biological system (such as serum triglycerides), and measurable physiological functions (such as a test of night vision or cognitive assessment) of future risk (9). This thesis focuses on biological biomarkers and metabolites measured in blood, specifically focusing on nutritional-related biomarkers and metabolites.

1.1.1 Nutritional-related biomarkers and metabolites

Nutritional-related biomarkers and metabolites reflect nutrient intakes and metabolism (10), and utilizing nutritional-related biomarkers and metabolites in blood offers several notable advantages. First, blood stands as a readily available source of fluid. It can be obtained in the routine care of patients, with minimally invasive procedures associated with little to no health risks to the patient. Furthermore, blood is considered homogenous compared to other biofluids, such as saliva or urine, which are influenced by the collection volume (11). One of the main advantages of utilizing biomarkers and metabolites in blood is that they are relatively cheap and easy to measure (12). The following section will introduce selected nutritional-related biomarkers and metabolites relevant to this thesis.

Glucose

Blood glucose concentrations are routinely measured in the clinic as an established biomarker for diabetes risk. It is well documented that glucose concentrations in blood are influenced by dietary intake; following food consumption, glucose concentrations

increase, resulting in insulin secretion from the pancreas, subsequently reducing blood glucose levels. Given the profound impact of dietary intake on blood glucose concentrations, it is common to distinguish between fasting and postprandial glucose concentrations, with fasting typically defined as no caloric intake within the last 8 hours. According to the American Diabetes Association, fasting (> 8 h) plasma glucose ≥ 7.0 mmol/L or plasma glucose concentrations ≥ 11.1 mmol/L two hours after an oral glucose tolerance test are diagnostic criteria for diabetes (13).

Amino acids

While more than 300 amino acids are found in nature, the human body relies on only 20 amino acids to form all the different proteins found in humans. These amino acids share a common structural framework, with an amino group and a carboxylic acid functional group, often referred to as the carbon skeleton. This distinctive carbon skeleton determines the unique property of each amino acid (14).

Beyond their functions as energy sources and components of proteins, amino acids are increasingly acknowledged for being involved in vital metabolic processes in the human body. These encompass the regulation of gene expression, cell signaling, antioxidative responses, immunity, and neurological and immunological functions (15). Given the amino acids´ integral roles in central pathways, the concentrations of amino acids in blood may change as a response to pathologic conditions (16-18). Consequently, plasma and serum amino acid profiles have received growing interest as potential biomarkers for a range of diseases, including various types of cancer (19), Alzheimer's disease (20), fatty liver disease (21), and diabetes (22). Notably, plasma levels of the branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) have emerged as particularly promising candidates for biomarkers for conditions and diseases such as diabetes, insulin resistance, cardiovascular disease, obesity, and metabolic syndrome (23). Nevertheless, despite the potential of amino acid profiles as biomarkers, their clinical implementation remains pending, with several unanswered questions needing resolution before they can be established as valid biomarkers in clinical practice (23-25).

One-carbon metabolites

The one-carbon metabolism comprises a series of interconnected metabolic pathways, including the folate cycle, the methionine-homocysteine cycle, the transsulfuration pathway, and the choline oxidation pathway. Transmethylation is a key reaction in one-carbon metabolism, wherein a one-carbon unit is transferred from a methyl donor to a methyl acceptor. These one-carbon units play a fundamental role in numerous cellular processes and are involved in both the synthesis and modification of various molecules (26, 27). Central one-carbon metabolites, some of them amino acids, include serine, glycine, methionine, methionine sulfoxide homocysteine, cystathionine, cysteine, dimethylglycine, sarcosine, choline, and betaine (28).

Perturbations in the one-carbon metabolism have been linked to various diseases, such as cardiovascular disease and cancer (28, 29). Consequently, the potential of one-carbon metabolites in blood as biomarkers has received attention. For instance, hyperhomocysteinemia is an established biomarker for various diseases, including cardiovascular disease, cancer, and diseases of the central nervous system, as well as impaired status of folate, vitamin B6, and cobalamin (30). Furthermore, increased serum and plasma levels of sarcosine have been suggested as a potential biomarker for prostate cancer (31, 32), while high levels of choline and low levels of betaine in plasma have emerged as potential biomarkers of risk of cardiovascular disease (33, 34). In patients with heart disease, elevated levels of dimethylglycine and cystathionine in plasma have been associated with an increased risk of acute myocardial infarction and mortality (35-38), while glycine and serine have received attention for their roles in cancer metabolism (29). However, apart from homocysteine, none of the other one-carbon metabolites are established biomarkers in the clinic.

Vitamin status

Vitamins are a group of organic compounds that must be obtained from the diet as they cannot be synthesized by the body, either as such or in sufficient amounts. They serve a diverse array of functions, and both deficiencies and excess of vitamins can adversely affect an individual´s health (39). However, vitamin intake is not a valid proxy for

vitamin status, and vitamin status may not always correlate directly with the biological effects or functions of that nutrient. Thus, suitable biomarkers are essential for accurately assessing vitamin status (9).

The B-vitamins are a group of eight essential water-soluble nutrients, with one of the main functions being coenzymes for several enzymatic processes involved in energy metabolism and one-carbon metabolism (40). Assessment of status differs across the different B-vitamins, and both direct and functional biomarkers exist. To assess thiamine (vitamin B_1) status, most laboratories measure blood thiamine concentrations directly (41). Although erythrocyte transketolase activity is considered the most reliable method for assessing thiamine deficiency, this test is not widely accessible outside research settings. The plasma concentration of thiamine monophosphate (TMP) has also been suggested as a biomarker for thiamine status (42). For assessing riboflavin (vitamin B_2) status, erythrocyte glutathione reductase is considered a functional biomarker of insufficient riboflavin intake, while plasma riboflavin concentrations tend to reflect recent dietary intake (41). The concentration of plasma flavin mononucleotide (FMN) is also suggested to be an indicator of riboflavin status (43). Niacin status (vitamin B_3) can be assessed by measuring urinary N-methylnicotinamide or erythrocyte NAD:NADP ratio; however, these tests are not widely available (41). For pyridoxine (vitamin B_6), the plasma level of pyridoxal-5´-phosphate (PLP) is most used to assess status (41, 44), while plasma levels of pyridoxal and 4´-pyridoxic acid are also suggested as indicators of status (44). Folate (vitamin B_9) status is usually assessed by measuring serum folate, while high homocysteine concentrations may also indicate impaired folate status (45). However, high homocysteine concentrations are not specific to folate deficiency, as homocysteine is also increased in the case of cobalamin (vitamin B_{12}) deficiency, among others. Cobalamin status is usually assessed by measuring a combination of biomarkers, including serum cobalamin, homocysteine, and methylmalonic acid (MMA) (46, 47).

The fat-soluble vitamins include the vitamins A, D, E, and K, each with essential functions in the body. Some examples of functions include that vitamin A is vital for vision (48), vitamin D is essential for bone health (49), and vitamin E has important

antioxidant properties (50). Vitamin K is found naturally as two vitamers: Vitamin K1 (known as phylloquinone) and K2 (known as menaquinone), both of which have major roles in coagulation pathways (51). Serum retinol is commonly used as the primary biomarker for vitamin A status. However, serum retinol concentrations are affected by infection and inflammation. Its concentrations are homeostatically regulated and do not correlate with liver stores of vitamin A unless vitamin A stores in the liver are depleted. Serum retinol-binding protein can be used as a proxy for serum retinol in the identification of vitamin A deficiency (48). For evaluating vitamin D status, serum levels of 25-hydroxyvitamin D concentrations are considered the most reliable indicator (49), while serum α-tocopherol levels usually assess vitamin E status. However, for patients with hyperlipidemia, α-tocopherol does not accurately reflect tissue vitamin levels and vitamin E status is estimated by the ratio between α-tocopherol and total blood lipids (50). Finally, assessing vitamin K status depends on the patient´s clinical signs and symptoms. Coagulation tests like prothrombin time or international normalized ratio are often used for bleeding patients, while vitamin K status can also be determined indirectly by measuring vitamin K-dependent factors such as prothrombin or coagulation factors. Phylloquinone levels can also be measured directly; however, this is impractical for clinical use (51).

Lipids

Dyslipidaemia is an established risk factor for cardiovascular disease. It is characterized by high blood levels of triglycerides, total- and low-density lipoprotein (LDL) cholesterol, and low levels of high-density lipoprotein (HDL) cholesterol (52). Lipid profile is widely measured in clinical care for cardiovascular risk assessment (53). Triglycerides, and total- and HDL cholesterol can be measured directly in blood, while LDL cholesterol can be either measured directly or calculated. As there is a lack of standardization across laboratories for measuring LDL cholesterol directly, the Friedewald equation has traditionally been used in clinical practice and trials for calculating LDL cholesterol. However, the Martin/Hopkins and Sampson equations have recently been suggested as alternative approaches for estimating LDL cholesterol (54).

Traditionally, lipid profiles have been obtained from fasting blood samples, where individuals refrain from caloric intake for a minimum of 8 hours before testing. This fasting period was implemented due to concerns that triglyceride concentrations increase in response to dietary intake, potentially influencing lipid profile results. However, emerging evidence has raised questions about the necessity of fasting for lipid profile assessment in cardiovascular risk evaluation. As a result, several clinical guidelines and expert consensus statements now open for non-fasting lipid testing for most clinical evaluations (53, 55).

Ketones

Ketones, or ketone bodies, refer to the molecules acetoacetate, β-hydroxybutyrate (βHB), and acetone (56). Ketones are synthesized mainly from acetyl coenzyme A (acetyl-CoA) generated by the β-oxidation of fatty acids, but some also originate from oxidation of the ketogenic amino acids (57). The major determinant for the production of ketones is the availability of fatty acids for oxidation. Mildly to moderately elevated levels of serum ketones are present in response to fasting, prolonged exercise, or when following a ketogenic diet low in carbohydrates and high in fats. Furthermore, elevated circulating serum ketone levels are frequently seen in newborn infants and during pregnancy (56). However, large increases in ketone levels can also be caused by pathological processes, such as diabetic ketoacidosis. In the clinic, ketone levels can be measured in blood, urine, and breath and are used as biomarkers for diabetic ketoacidosis (58). Moreover, elevated levels of plasma ketones have recently been explored as biomarkers for other conditions, such as heart failure (59).

Acylcarnitines

Acylcarnitines are esters formed through the conjugation of fatty acids with carnitine, and their primary role is to transport fatty acids across the mitochondrial membrane for β-oxidation. Acylcarnitines can be broadly classified into four groups based on the number of carbon atoms in the acyl-chain: Short-chain (C2-C5), medium-chain (C6-C12), long-chain (C13-C20), and very long-chain (>C21) acylcarnitines. Long-chain acylcarnitines are measured in newborn screening to identify fatty acid oxidation

disorders. Additionally, blood levels of acylcarnitines have been associated with a range of diseases, such as cardiovascular disease, diabetes, depression, neurological disorders, and cancer (60). However, despite their potential as biomarkers for various diseases, the routine measurement of acylcarnitines in clinical practice extends primarily to newborn screening. Further robust translational work is imperative to fully utilize the potential of acylcarnitines as biomarkers (61). This research should delve into aspects such as the natural variability in acylcarnitine concentrations under normal physiological conditions (62).

1.1.2 Measurement considerations

When a blood biomarker or metabolite is measured in the laboratory, the results are always under the influence of uncertainty. Measuring a biomarker or metabolite repeatedly would likely lead to different results for the repeated measures, although the individual's health status is the same. Sources of test result variation may be broadly categorized into preanalytical, analytical, and within-person biological variation (63).

Preanalytical variation

The preanalytical process encompasses the preparation before blood sampling, collection of the blood sample, and handling, transport, and storage of the blood sample before measurement (64). While this process is commonly described as the “easy part” of laboratory testing, small variations in preanalytical procedures may affect the blood sample quality (65). For example, it has been shown that the duration of tourniquet application may introduce variability in the measurement of several metabolites in plasma (66) and that prolonged exposure of whole blood to room temperature before centrifugation affects certain metabolites such as amino acids (67). Moreover, storage of whole blood with ice for up to 48 hours before processing has been shown to introduce variations in several B-vitamers and amino acids in plasma (68, 69) and serum (69). The choice of sample matrix may also influence biomarker and metabolite assessment. For instance, serum samples usually exhibit higher levels of most metabolites compared to plasma samples (70-74). In addition, the type of anticoagulant added to obtain plasma samples (*e.g.* Ethylenediaminetetraacetic acid [EDTA],

heparin, citrate) has been shown to introduce variations in several amino acids, ketones, and some one-carbon metabolites (75).

Analytical variation

Every analytical technique has some intrinsic sources of variability which cannot be eliminated. The analytical variation is of two types: Random and systematic variation. Random variation refers to the closeness of agreement between independent results of a measurement. Random variation can arise from sources such as fluctuations in temperature, changes in the environment, variability in the volume of a sample, and inconsistent handling of a material. If a method has good precision, its random variation is low, and the results obtained with this method will not change much over time due to analytical influences. The random variation of an analytical technique can be quantified by the standard deviation (SD) and the coefficient of variation (CV), which is calculated as ($^{SD}/_{mean} \times 100$) and expressed as % (64).

Systematic variation is also referred to as bias and is the difference between the observed result and the estimate of the true value of the biomarker or metabolite. Bias can be caused by factors such as issues related to the calibration or validation of analytical methods or if individuals perform tasks consistently but differently from others during the analysis. If bias is present, there will be a consistent over- or underestimation of the true value of a measurement. Constant bias itself will not contribute to variation in the laboratory results over time. However, if major changes are made to instruments or methodology to reduce bias, and if samples from an individual are compared before and after such changes, differences in bias may result in increased variability in the test results over time (64).

Biological variation

In theory, if one could remove all sources of variations in the pre-analytical and analytical phases, repeated measures of a biomarker or metabolite would still vary within an individual. This is because the human blood metabolome is highly variable and influenced by internal and external factors (5, 76, 77). First, biomarker and metabolite concentrations may fluctuate randomly around a homeostatic setting point

within an individual. This is termed within-subject biological variation. Further, this homeostatic setting point varies between individuals, referred to as between-subject biological variation (64).

However, biomarkers and metabolites in blood may also vary in non-random ways. These non-random variations may be due to internal factors such as growth, aging, pregnancy, menopause, or health and disease status (78). Information on the biological changes that occur throughout life can be used to stratify reference values, for instance, according to age (79). Biological variability may also be related to predictable cyclic variations. Knowledge of the predictable biological cyclic rhythms within individuals is paramount when interpreting the results of a laboratory measurement (79-81). These cyclic variations can be seasonal, such as for 25-hydroxyvitamin D, where population concentrations in countries far from the equator tend to reflect changes in ultraviolet β radiation from the sun (82). Certain biomarkers and metabolites may also exhibit daily variations, such as those governed by diurnal rhythm. One example is the stress hormone cortisol, which follows a circadian rhythm with peak levels just before waking up in the morning and the lowest levels during sleep around midnight (83).

The metabolic response to dietary intake and fasting is a source of short-term biological variations in biomarkers. Following food consumption, biomarker concentrations can rapidly change, necessitating considerations of prandial status when evaluating certain biomarkers. For example, to improve the sensitivity and specificity of glucose as a biomarker of diabetes, different cutoff values are established for glucose levels depending on whether measurements were taken from fasting or non-fasting blood samples (13). Furthermore, prandial status is also acknowledged when measuring blood lipid profiles (53, 55). However, in contrast to glucose, less is known about how the concentrations of other nutritional-related biomarkers may change during the metabolic response to dietary intake and fasting.

1.2 Human energy metabolism

Energy metabolism refers to the process of generating energy in the form of adenosine triphosphate (ATP) from nutrients. A simplified overview of the main pathways of energy metabolism and the connection with selected nutritional-related biomarkers and metabolites relevant to this thesis is provided in **Figure 1**. In the period after a meal, known as the postprandial state, the energy is generated from the macronutrients obtained from the food. In contrast, the energy must be released from the body´s internal stores during fasting. This section will describe the postprandial and fasting energy metabolism.

1.2.1 Postprandial energy metabolism

The postprandial state represents the metabolic response to a meal (84) and embodies the digestion and absorption of nutrients. As the dietary pattern in the Western world usually involves several meals during a day, it is estimated that healthy individuals in Western countries spend most of their awake time (~18 hours a day) in the postprandial state. Following is a description of the metabolism of the macronutrients carbohydrates, protein, and fat.

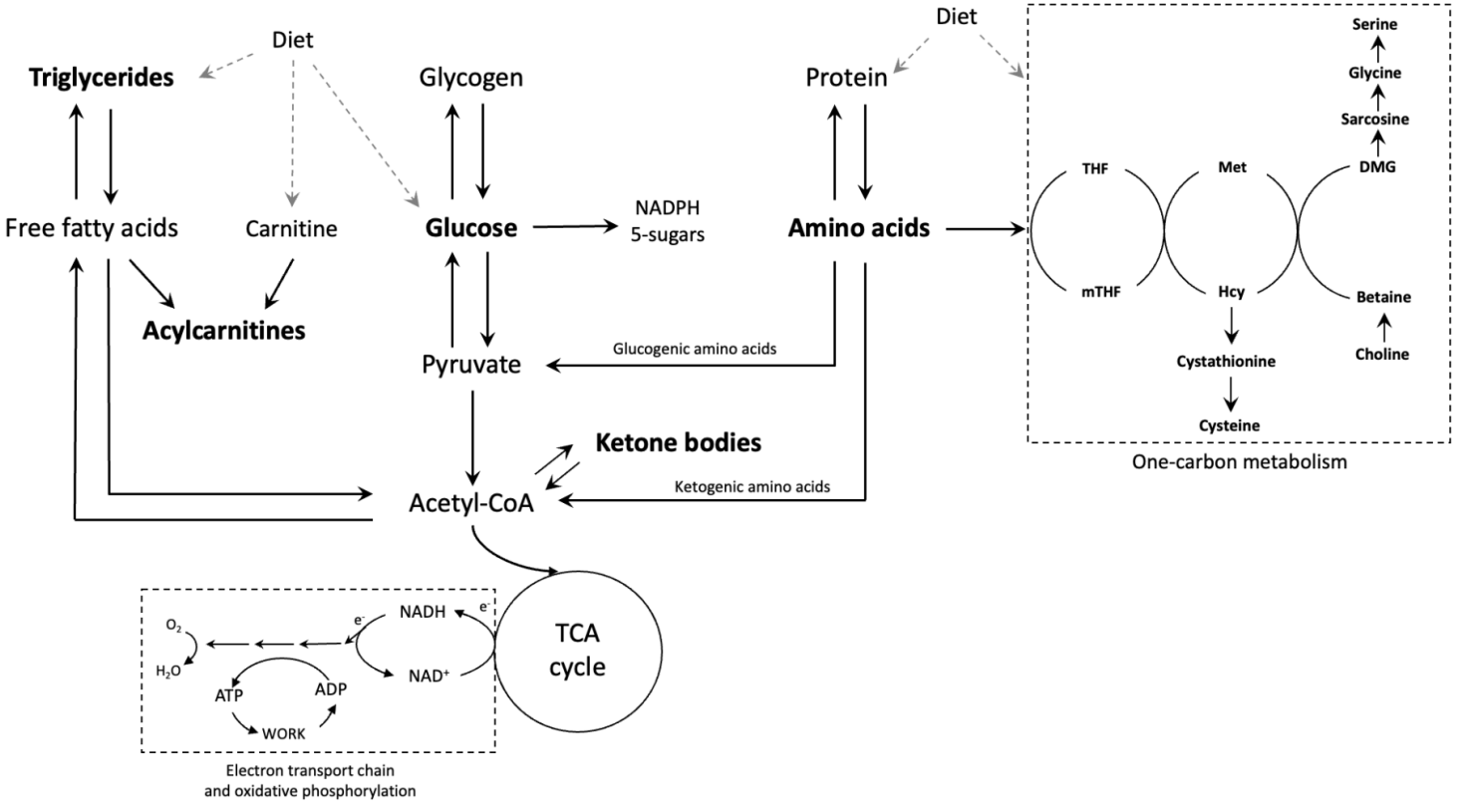

Figure 1. A simplified overview of the energy metabolism and the link with selected biomarkers and metabolites relevant for this thesis. **Abbreviations**: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, Coenzyme A; DMG, dimethylglycine; Hcy, homocysteine; Met, methionine; mTHF, 5-methyltetrahydrofolate; NAD, nicotinamide adenine dinucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; THF, Tetrahydrofolate; TCA, tricarboxylic acid

Carbohydrates

Carbohydrate metabolism centers around glucose, and glycolysis and glycogen synthesis are the dominating metabolic pathways involving glucose metabolism after a meal. Carbohydrate digestion results in the breakdown into the monosaccharides glucose, fructose, and galactose, which are subsequently absorbed in the small intestine and transported via the portal vein to the liver (85, 86). Here, galactose and fructose are metabolized and converted to glucose (87), and once within the hepatocyte, glucose is rapidly phosphorylated to form glucose-6-phosphate (88). Glucose-6-phosphate has three fates: It may enter the pathway of glycolysis to form pyruvate, form glycogen through the pathway of glycogen synthesis, or enter the pentose phosphate pathway for synthesis of nucleotides, among others (89). In the postprandial state, when glucose is abundant, insulin is secreted and stimulates glycolysis. The end-product of glycolysis is pyruvate, which is further converted to acetyl-CoA. Acetyl-CoA can enter the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the electron transport chain or be used to synthesize fatty acids through lipogenesis (88). Furthermore, insulin also activates the storage of glucose as glycogen, which provides an immediately available reserve of glucose to maintain blood glucose concentrations (90). Finally, glucose-6-phosphate may be metabolized via the pentose phosphate pathway, where ribose-5-phosphate is generated to be used for the synthesis of nucleotides, as well as nicotinamide adenine dinucleotide phosphate (NADPH), which is used for biosynthesis of fatty acids and cholesterol, among other. The pentose phosphate pathway is a metabolic parallel to glycolysis; however, it has a much lower flux than glycolysis (91).

The primary function of carbohydrate metabolism is to maintain glucose concentrations in blood in a tight physiological range close to 5 mmol/L, as both low (<3 mmol/L) and consistently elevated (>11 mmol/L) concentrations may have harmful effects (89, 90). After a meal containing carbohydrates, glucose concentrations increase and usually peak at 7-8 mmol/L around 30-60 minutes after the meal in healthy individuals. The liver is often described as acting like a buffer for glucose: It takes up glucose and stores it as glycogen when the portal concentration of glucose is high, such

as after ingesting a meal, and releases glucose when it is required elsewhere in the body (89).

Fats

Dietary fat is almost entirely in the form of triglycerides, which are incorporated in chylomicrons within the enterocytes and enter the plasma via the lymphatic system (92). Most of the triglycerides are readily removed from the chylomicrons in adipose tissue, skeletal muscle, and the heart. These tissues contain lipoprotein lipase (LPL), which hydrolyses the triglycerides into glycerol and free fatty acids. Insulin, which increases after a meal, stimulates the activity of LPL. In adipose tissue, LPL hydrolyses triglycerides to form free fatty acids, which enter the adipocytes and are re-esterified to form new triglycerides for storage. However, in cardiac and skeletal muscles, the free fatty acids generated by LPL are mostly oxidized through β-oxidation (89).

The process of absorption of triglycerides is slower than the absorption of glucose. Thus, plasma triglyceride usually peaks around three to five hours after a fatty meal. While an overnight-fasted plasma triglyceride concentration may be around 1 mmol/L, the concentrations may rise to ~2 mmol/L after a particularly fatty meal (89). The postprandial peak triglyceride concentrations are affected by several factors, including meal size and composition. A normal meal, containing typically 30-40 grams of fat, is sufficient to raise the plasma triglyceride concentrations. Furthermore, consecutive meals containing fat appear to enhance this peak, known as the second meal effect (93). Thus, the typical dietary pattern of consumption in the Western world, involving frequent meals and snacks between meals, might be expected to maintain circulating triglyceride levels above fasting concentrations for much of the day (92).

Protein

After protein ingestion, proteins are hydrolyzed to free amino acids, dipeptides, and tripeptides for absorption. Amino acids are mostly actively absorbed by sodium-linked carriers, while some di- and tripeptides are absorbed intact by peptide transporters. Inside the enterocyte, peptidases cleave the peptides into amino acids, which are

transported to the liver via the portal vein. In a typical Western diet, one eats approximately 100 grams of protein daily. As the total amount of protein in the body is steady from day to day in healthy individuals, one must dispose of an equal amount of protein, mainly by oxidation of amino acids. Although there is a net overall protein synthesis after a meal, and some amino acids are used for protein synthesis or enter the systemic circulation, most of the amino acids ingested are ultimately oxidized in the liver to yield energy (94). Amino acids contain an amino group that must be removed before amino acids can produce energy. The removal of the amino group is called *deamination* and produces ammonia (NH_3) and the carbon skeleton of the amino acids. Ammonia is toxic and must be excreted by the urine or converted to non-toxic urea in the urea cycle, while the carbon skeleton can be used for energy production. Deamination is achieved by two reactions: *Transamination* and *oxidative deamination*. In the transamination reaction, the amino group from an amino acid is transferred to an acceptor ketoacid, generating the corresponding amino acid and a new ketoacid. Most amino acids donate their amino group to the keto acid α-ketoglutarate, generating glutamate and a new keto acid. The new keto acid undergoes metabolism and ultimately produces energy by a route depending on its structure; the carbon skeletons of the glucogenic amino acids are metabolized to TCA intermediates, while the carbon skeletons of the ketogenic amino acids are metabolized to acetyl-CoA. Glutamate, formed during transamination, undergoes oxidative deamination, producing ammonia and regenerating α-ketoglutarate (89).

Although most amino acids are ultimately oxidized in the liver after a meal, some amino acids also enter the systemic circulation. Thus, plasma concentrations of amino acids usually increase after a meal (95). However, while the amino acids in the portal vein largely reflect the amino acid composition of the meal, the amino acids leaving the hepatic vein show different proportions. For instance, the BCAAs leucine, isoleucine, and valine constitute about 20% of the dietary protein; however, they represent about 70% of the amino acids leaving the liver after a meal (89). Furthermore, plasma amino acid concentrations reflect the balance between several processes, including amino acid release from tissues, synthesis of new amino acids, loss of amino

acids by incorporation into proteins, amino acid oxidation, and conversion to other metabolites. Therefore, plasma amino acid concentrations after a meal cannot be used to quantify the amount of exogenous amino acids absorbed (96, 97).

1.2.2 The metabolic transition between the postprandial and fasting state

The duration of the postprandial state is not precisely defined, as it varies depending on several factors, including meal size and composition, as well as energy expenditure, which is further influenced by body mass and -composition and physical activity levels (89). For instance, for meals high in glucose, the postprandial period would be around 2-3 hours, whereas for meals high in fats, the postprandial period could be up to eight hours (98). The literature also reflects this variability in the definition of the postprandial state, with different timeframes used to define the postprandial period. For example, the term "postprandial period" has been applied to durations ranging from 90 minutes to 6 hours (99-103). Furthermore, the postprandial and fasting states are often referred to as clearly separated metabolic states. However, the transition between the postprandial and fasting states occurs gradually, with a gradual metabolic transition from using glucose as the primary energy substrate in the postprandial state towards fatty acids becoming the main source in the fasting state. Given the variability in the duration of the postprandial period and the gradual transition between the postprandial and the fasting states, it is crucial to note that there is no clear or universally agreed-upon precise moment for when one leaves the postprandial state and enters the fasting state.

1.2.3 Fasting energy metabolism

As the dietary pattern in the Western world typically involves several meals during the day, healthy individuals usually enter the fasting state only after an overnight fast before breakfast is consumed (89). The fasting state may also be called the *postabsorptive state* and implies the state in which the last meal has been digested and absorbed from the intestinal tract. In this state, the body is mobilizing stored energy reserves. This section will provide a brief overview of the energy metabolism in the body during the fasting state.

Glucose

In the overnight fasted state, glucose typically supplies about one-third of the body´s energy requirements. In this state, glucose enters the blood almost exclusively from the liver, originating mainly from glycogen breakdown and gluconeogenesis (94). It is estimated that about half of the glucose originates from glycogen breakdown and the other half from gluconeogenesis, however, with large variations depending on glycogen storage, which in turn depends on previous diet and exercise. Furthermore, the longer the fast continues, the more glucose will originate from gluconeogenesis. Both glycogen breakdown and gluconeogenesis are stimulated by decreased levels of insulin and increased levels of glucagon (89, 104). The main substrates for gluconeogenesis are lactate, alanine from muscles, and glycerol from adipose tissue lipolysis (89).

Lipids

Lipids are the main supply of energy to the body after an overnight fast. When insulin levels are low, the enzyme hormone-sensitive lipase is activated and stimulates lipolysis of triglycerides in adipose tissue, releasing glycerol and free fatty acids. Glycerol is used as a substrate for gluconeogenesis in the liver, while the free fatty acids are taken up predominantly by the liver and skeletal muscle, where they are oxidized through β-oxidation. Acetyl-CoA generated from the β-oxidation may either generate energy through the TCA cycle or enter the ketogenesis pathway (89).

Amino acids

As opposed to the postprandial period, wherein there is an overall net protein synthesis, there is a net breakdown of protein after an overnight fast. Oxidation of amino acids contributes to about 10-15% of energy production after fasting overnight (94). Furthermore, when glycogen stores are depleted, the glucogenic amino acids are important substrates for gluconeogenesis to provide glucose needed by the brain, renal medulla, and erythrocytes. During longer-term fasting, the ketogenic amino acids are also used to synthesize ketones (89).

Ketones

During prolonged fasting, when glucose is not readily available, ketones become an important alternative source of energy. Ketones are distributed via the blood to tissues, most importantly by the brain, where they are metabolized to be used for energy (105). Most of the ketone levels are in the form of βHB, which is typically present in blood about five times the concentration of acetoacetate (106). During a typical day, their concentrations in blood are relatively low, usually ~100-250 μmol/L for βHB and acetoacetate combined (57, 89). However, after 24 hours of fasting, their concentrations rise to about 1000 μmol/L (57) and contribute to about 2-6% of the body´s energy requirements (56).

1.3 The dynamics of biomarkers and metabolites during the postprandial and fasting states

The metabolic response to dietary intake and fasting can introduce variations in blood biomarkers and metabolites. Apart from well-established nutritional-related biomarkers such as glucose, there is limited knowledge of the dynamics of circulating concentrations of other nutritional-related biomarkers and metabolites during the postprandial and fasting states. However, a few studies have investigated the dynamics in blood biomarkers and metabolite concentrations related to glucose-, lipid-, and amino acid metabolism.

1.3.1 The postprandial state

In 2021, LaBarre and colleagues published a review on the effect of a mixed-macronutrient challenge on metabolites related to the amino acid-, glucose-, and lipid metabolism, and ketones up to 8 hours after the challenge (95). In this review, including 26 articles, they reported that the blood concentrations of amino acids tended to peak 60-90 minutes after the test meal, returning to baseline values at later time points, except for glutamate and aspartate, which decreased shortly after the meal. They also reported a consistent decrease in levels of free fatty acids and other lipid classes

between baseline and 120 minutes after the meal, parallel with reduced concentrations in ketones right after the meal (95). In addition, they reported that the medium- and long-chain acylcarnitines decreased, while propionylcarnitine and valerylcarnitine increased in response to the mixed-macronutrient challenge.

1.3.2 The fasting state

The fasting state also poses changes in the human metabolism, which may affect circulating biomarker concentrations, and a few studies have characterized the dynamics in biomarker concentrations during the fasting state. In 2011, Rubio-Aliaga and colleagues (107) reported an analysis of the "human fasting metabolome". In this study, three males and seven females fasted for 36 hours, and blood samples were taken at 12 and 36 hours of fasting. They observed that most amino acids decreased from 12 to 36 hours of fasting, with methionine showing the most pronounced change (50% decrease). On the contrary, the BCAAs increased in this period. They also observed changes in acylcarnitine concentrations, with most acylcarnitines (70%) increasing while free carnitine decreasing from 12 to 36 hours. In 2012, Krug and colleagues (108) investigated biomarker changes in response to 36 hours of fasting in 15 healthy males. In this study, participants were served a standardized evening meal at 7 p.m. and thereafter fasted for 36 hours. Blood samples were drawn at 8 a.m. the next morning, and then every other hour until midnight (29 hours fasting), with a last blood sample after 36 hours of fasting. Like Rubio-Aliaga (107), Krüg et al. also reported that the BCAAs and most acylcarnitines, except for the short-chain acylcarnitines, increased until 36 hours of fasting, while free carnitine decreased. In a small study of four participants, Teruya and colleagues (109) quantified blood biomarkers after 10, 34, and 58 hours of fasting. Like the aforementioned studies, they reported that the BCAAs and several acylcarnitines increased during fasting.

2. Knowledge gaps and rationale for this thesis

Dietary intake and fasting induce major metabolic changes in human energy metabolism, which can be a source of variations in nutritional-related blood biomarkers and metabolites. Consequently, to account for prandial status, it is common in clinical practice and research settings to distinguish between non-fasting and fasting blood samples by applying a given cut-off, usually 8 hours since the last caloric intake. While this approach may be convenient, it does not account for the gradual transition between the two states, which exists on a continuum without a clear cutoff point. Furthermore, the blood metabolome is dynamic within both the postprandial and fasting states and blood biomarker and metabolite concentrations can fluctuate within the two states. It is likely that categorizing blood samples as non-fasting or fasting does not fully capture the potentially dynamic nature of blood biomarkers and metabolite concentrations and may lead to measurement error and misclassification due to within-person biological variation. Therefore, knowing how biomarker concentrations may change dynamically during the postprandial and fasting states is imperative.

The dynamics in biomarker and metabolite concentrations related to glucose-, lipid-, and amino acid metabolism have been explored to some extent previously. However, beyond well-established nutritional-related biomarkers such as glucose, there is still limited knowledge about the dynamics of circulating concentrations of other nutritional-related biomarkers and metabolites after dietary intake and during fasting. Unraveling how the concentration of these biomarkers and metabolites may change during the postprandial and fasting states, and whether these changes are influenced by factors such as age and sex, can enhance our ability to interpret and utilize these biomarkers and metabolites effectively in both clinical care and research.

3. Objectives

The overall objective of this thesis was to investigate the dynamics of nutritional-related blood biomarker and metabolite concentrations in the hours following dietary intake and during the adaptation to the fasting state, focusing on the first 24 hours after a meal. Furthermore, the aim was to explore potential age and sex differences in the change in biomarker concentrations.

3.1 Specific study objectives

Paper I: To provide a descriptive overview of the concentrations of plasma amino acids, one-carbon metabolites, biomarkers of vitamin status, and serum lipids in the first 7 hours after habitual dietary intake in middle-aged and elderly adults, and to explore potential age- and sex differences.

Paper II: To investigate the changes in serum concentrations of amino acids, one-carbon metabolites, and B-vitamin biomarkers during 24 hours after a standardized meal in healthy, young individuals, and the potential influence of sex.

Paper III: To investigate the changes in serum concentrations of lipids, ketones, and acylcarnitines during 24 hours after a standardized meal in healthy, young individuals, and the potential influence of sex.

4. Materials and methods

4.1 Study populations, design, and data collection

To meet the aims of this thesis, two distinct data sources were used: Cross-sectional data from the Hordaland Health Study (hereafter referred to as "HUSK") and time series data from the interventional Postprandial Metabolism (PoMet) study. An overview of the key characteristics of the papers included in this thesis is provided in **Table 1.**

Table 1. An overview of the key characteristics of the included papers

	Paper I	**Papers II and III**
Project	HUSK	PoMet
Sample size	*n* = 5835	*n* = 34
Ages	46-49 and 70-74 years	20-30 years
Biomarker and metabolite data	Cross-sectional	Time series
N blood sampling timepoints	1	14
Sample matrix	EDTA plasma and serum (lipids only)	Serum
Duration	7 hours after meal	24 hours after meal
Meal prior to blood sampling	Not standardized	Standardized
Time of day at blood sampling	Not standardized	Standardized

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; HUSK, Hordaland Health Study; PoMet, Postprandial Metabolism Study

3.1.1 Paper I: The Hordaland Health Study

Paper I was based on data from the first visit (baseline data) from the observational community-based HUSK study (https://husk.w.uib.no/), wherein the baseline measurements were conducted in 1997-99 in Bergen, Norway. The data collection in the HUSK study was conducted as a collaboration between the National Health Screening Service (now the Norwegian Institute of Public Health), the University of Bergen, and local health services. The cohort consisted of individuals aged 46-49 years (born in 1950-51, referred to as the "middle-aged group", n = 3090) and individuals aged 70-74 years at baseline (born in 1925-27, referred to as the "elderly group", n = 2969) who were living in the city of Bergen or neighboring suburban municipalities. The study design and methodology have been described in more detail elsewhere (110).

Study participants

A flowchart illustrating the inclusion and exclusion process of participants is shown in **Figure 2**. We excluded participants with missing information on time since the last meal and participants with ≥ 7 hours since the last meal. This left us with a total of 5834 participants: 2960 participants in the middle-aged group, and 2874 participants in the elderly group.

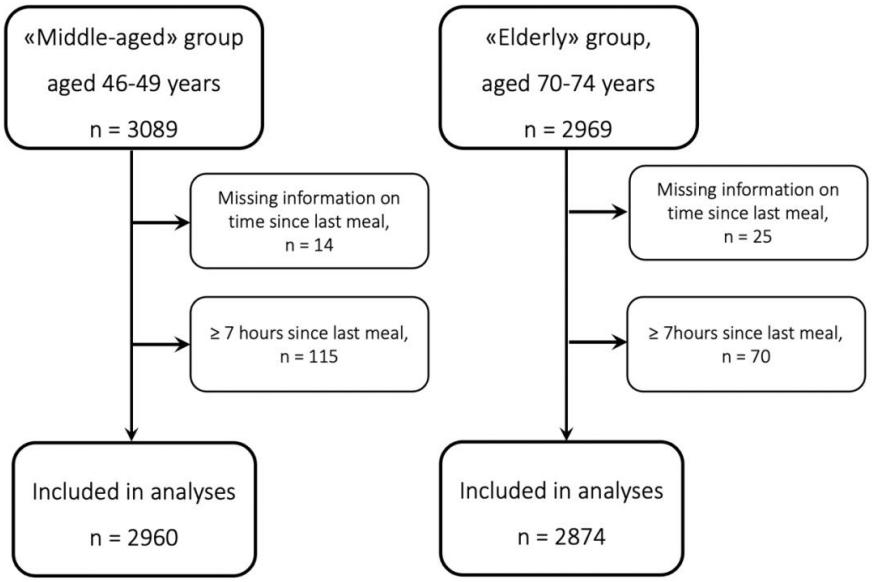

Figure 2. Flowchart illustrating the inclusion and exclusion process of participants in the two age cohorts in the Hordaland Health Study 1997-1999

Blood sampling and handling of the blood samples

Blood sampling was conducted at the first visit, which was held between 8 a.m. and 6 p.m. Blood samples were collected only once from each participant, and the participants attended the first visit and provided blood samples with a different number of hours since the last meal. The number of hours since the last meal was registered at the time of blood sampling and categorized into hourly categories. The time categories were given as: (1) 0 – < 1 hour, (2) 1 – < 2 hours, (3) 2 - < 3 hours, (4) 3 - < 4 hours, (5) 4 - < 5 hours, (6) 5 - < 6 hours, and (7) 6 - < 7 hours after a meal.

Serum was obtained by collecting blood into Vacutainer Tubes with no additive. Blood was allowed to clot at room temperature for 30 minutes before isolation of the serum fraction. Plasma samples were collected into evacuated tubes containing EDTA, chilled at 4-5°C within 15-30 minutes, and then centrifuged at 4000 x *g* at 10°C for 10 minutes within one to three hours. Aliquots of serum and plasma were stored at -80°C until analysis.

3.1.2 Papers II and III: The Postprandial Metabolism (PoMet) study

Papers II and **III** were based on data from the PoMet study, which was an intervention study carried out in Bergen, Norway, during the autumn of 2021. During the study visit, participants were served a standardized breakfast meal, and blood samples were taken before and at 13 standardized time points from 15 minutes to 24 hours after the breakfast meal. In these 24 hours, participants were not allowed to consume anything other than water.

Sample size calculation

The sample size calculation for the PoMet study was performed using an accuracy-in-parameter-estimation (AIPE) approach, as recommended when the main purpose is to accurately estimate the parameters of interest (20, 21). For the main analysis, we aimed to achieve a multiplicative margin-of-error (gSE1.96) < 1.10, corresponding to a geometric standard error (gSE) < 1.05 for at least 80% of the measurements. Using freely available data on 132 metabolites across 56 timepoints (7392 estimates) across different metabolic challenges from the HuMet study (108) (available from http://metabolomics.helmholtz-muenchen.de/humet/), the observed median (80th percentile) geometric standard deviation (gSD) was 1.24 (1.32). Rearranging equation 1 above, and solving for n with a gSD = 1.32, it was estimated that we needed a sample size of 32 to achieve the desired precision level. Precision curves as a function of sample size are provided in **Figure 3A**. The expected distribution of multiplicative margin-of-errors with a sample size of 32 is illustrated in **Figure 3B**, based on repeated resampling with replacement from HuMet (50 replications, 369 600 simulated estimates). Due to previous experience with similar studies using a venous catheter to draw repeated blood samples, we expected a drop out of up to 10% due to adverse events following fasting blood sampling or difficulties drawing blood from a venous catheter. Therefore, to achieve our goal of collecting complete data for 32 participants, we aimed to recruit a total of 36 participants (18 males and 18 females).

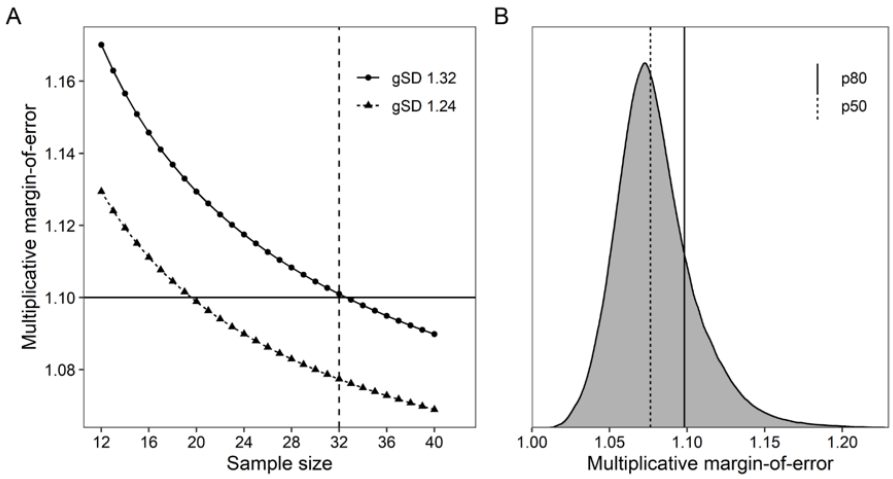

Figure 3. A) Precision curves as a function of sample size, using the observed median (20th-80th percentile) geometric standard deviations from the HuMet study (available from http://metabolomics.helmholtz-muenchen.de/humet/). **B)** The expected distribution of multiplicative margin-of-errors for the measurements of different metabolites at different time points, with n = 32. We expected to be able to estimate the geometric mean concentrations within a multiplicative margin-of-error of 1.10 for at least 80% of all measurements. **Abbreviations:** gSD, geometric standard deviation

Recruitment

Participants for the PoMet study were recruited through social media channels, snowball sampling, and posters in the nearby area. In short, the aim was to recruit a homogenous group of healthy participants similar in age, body mass index (BMI), and health status. Inclusion criteria for participation were aged 20-30 years (birth years 1991 – 2001) and a BMI between 22-27 kg/m^2. Individuals were excluded if they had experienced acute or chronic disease such as diabetes, thyroid diseases, cancer, cardiovascular disease, or inflammatory bowel disease during the last three years; had celiac disease or other food allergies interfering with the standardized breakfast meal; used any prescription medications except for contraceptives; smoked or used other nicotine-containing products such as “snuff” regularly; were pregnant or had breastfed

during the last three months before the study visit; or had experienced significant weight change (> 5%) during the last three months before the study visit.

Study participants

A flowchart depicting the inclusion of participants is illustrated in **Figure 4.** Initially, 18 males and 18 females were enrolled in the study. Two females withdrew from the study due to difficulties in blood sampling and were excluded from all analyses. One female completed the first two hours of blood sampling and withdrew thereafter due to difficulties with blood sampling. This participant was included in the analyses. In total, 33 participants completed the study, while data from 34 participants (18 males and 16 females) were included in the analyses.

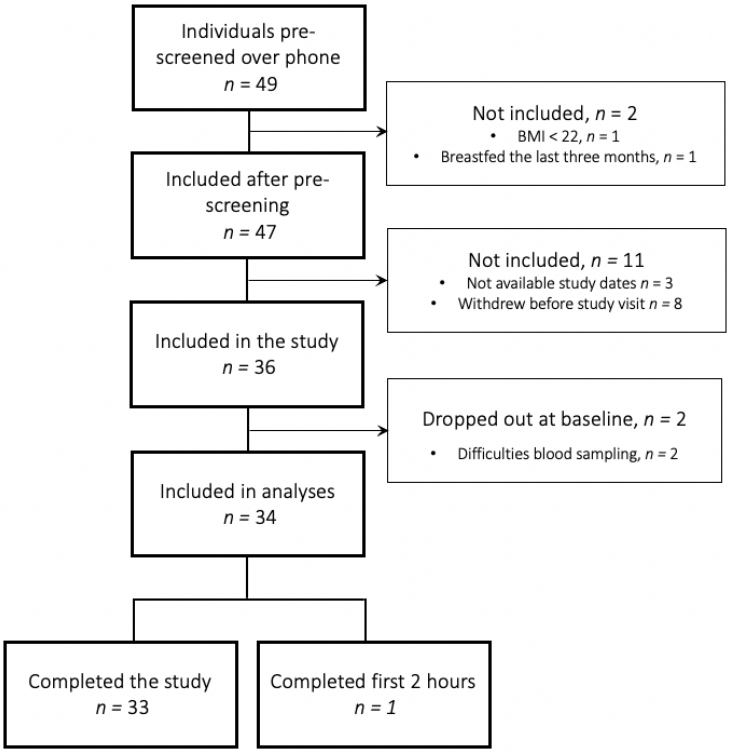

Figure 4. Flowchart of the inclusion process for participants in the Postprandial Metabolism Study

The study visit

The course of the study visit is illustrated in **Figure 5**. At the study visit, participants received a standardized breakfast meal, similar to a "standard Norwegian breakfast". The breakfast consisted of three slices of bread with butter, cheese, jam, and a glass of orange juice and contained ~500 kcal, 20 grams fat (36E%), 60 grams carbohydrates (46E%), 5 grams dietary fiber (2E%), and 20 grams protein (16 E%). The participants were instructed to consume the breakfast in precisely 15 minutes. Blood samples were taken of the participants before the breakfast and at 15, 30, 45, 60, 90, and 120 minutes, 3 hours, 4 hours, and thereafter every other hour until 12 hours after the meal. After the 12-hour blood sample, the participants left the study center overnight and returned the following day for the last blood sample taken 24 hours after the breakfast meal. During these 24 hours, the participants were instructed to consume nothing but water and to avoid strenuous activity and the use of nicotine-containing products.

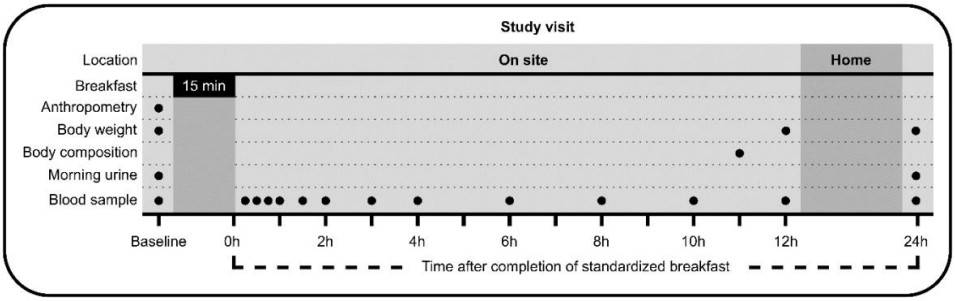

Figure 5. The course of the study visit in the Postprandial Metabolism Study

Blood sampling and handling of the blood samples

The first 12 blood samples were drawn through a venous catheter in the elbow cavity, while the last two were standard venous blood samples. At each time point, a total of 11.5 ml of blood was drawn and distributed into serum tubes (8.5 ml, BD Vacutainer® SST™ II *Advance*; Beckton, Dickinson, and Company; United Kingdom) and EDTA tubes (3ml, Vacuette® K2EDTA). At baseline and at the 24-hour timepoint, an

additional 6 ml and 3 ml EDTA blood was collected, respectively, for measurement of hematology and routine clinical markers.

After the blood sampling, the serum tubes were stored at room temperature for 30-60 minutes and then centrifuged at 2200xG for 10 minutes at 20ºC. The serum tubes were temporarily stored in a freezer at -20ºC and transferred to -80ºC at the end of the day. Additionally, one aliquot of serum from each time point was stored in the refrigerator at +4ºC and transported to the laboratory daily for analysis of blood lipids, folate, cobalamin, and routine clinical markers (liver and kidney function biomarkers, albumin, C-reactive protein, erythrocytes, hemoglobin, hemoglobin A1c (HbA1c), insulin, mean corpuscular hemoglobin, mean corpuscular volume, mean platelet volume, thrombocytes, thyroid stimulating hormone, and 25-hydroxyvitamin D).

4.2 Quantification of outcome biomarkers and metabolites

An overview of the outcome metabolites in the three papers, together with the analytical method and sample matrix, is provided in **Table 2.**

Table 2. Overview of the outcome metabolites, sample matrix, and the analytical methods across the papers[1]

Biomarker	Paper I	Paper II	Paper III
Amino acids	**Plasma**	**Serum**	**-**
Alanine	GC-MS/MS	GC-MS/MS	-
Arginine	GC-MS/MS	LC-MS/MS	-
Asparagine	GC-MS/MS	GC-MS/MS	-
Aspartic acid	GC-MS/MS	GC-MS/MS	-
Glutamic acid	GC-MS/MS	GC-MS/MS	-
Glutamine	GC-MS/MS	GC-MS/MS	-

Biomarker	Paper I	Paper II	Paper III
Histidine	GC-MS/MS	GC-MS/MS	-
Isoleucine	GC-MS/MS	GC-MS/MS	-
Leucine	GC-MS/MS	GC-MS/MS	-
Lysine	GC-MS/MS	GC-MS/MS	-
Phenylalanine	GC-MS/MS	GC-MS/MS	-
Proline	GC-MS/MS	GC-MS/MS	-
Threonine	GC-MS/MS	GC-MS/MS	-
Tryptophan	GC-MS/MS	GC-MS/MS	-
Tyrosine	GC-MS/MS	GC-MS/MS	-
Valine	GC-MS/MS	GC-MS/MS	-
One-carbon metabolites	**Plasma**	**Serum**	-
Betaine	LC-MS/MS	LC-MS/MS	-
Choline	LC-MS/MS	LC-MS/MS	-
Cystathionine	GC-MS/MS	GC-MS/MS	-
Cysteine	GC-MS/MS	GC-MS/MS	-
Dimethylglycine	LC-MS/MS	LC-MS/MS	-
Glycine	GC-MS/MS	GC-MS/MS	-
Homocysteine	GC-MS/MS	GC-MS/MS	-
Methionine	GC-MS/MS	GC-MS/MS	-
Methionine sulfoxide	-	LC-MS/MS	-
Sarcosine	-	GC-MS/MS	-
Serine	GC-MS/MS	GC-MS/MS	-
B-vitamin biomarkers	**Plasma**	**Serum**	-
Thiamine	LC-MS/MS	LC-MS/MS	-
TMP	LC-MS/MS	LC-MS/MS	-

Biomarker	Paper I	Paper II	Paper III
Riboflavin	LC-MS/MS	LC-MS/MS	-
FMN	LC-MS/MS	LC-MS/MS	-
NAM	LC-MS/MS	LC-MS/MS	-
mNAM	LC-MS/MS	LC-MS/MS	-
Pyridoxal	LC-MS/MS	LC-MS/MS	-
PLP	LC-MS/MS	LC-MS/MS	-
PA	LC-MS/MS	LC-MS/MS	-
Folate	Microbiological assay	Immunoassay[2]	-
Cobalamin	Microbiological assay	Immunoassay[2]	-
MMA	GC-MS/MS	GC-MS/MS	-
Lipid-soluble vitamin biomarkers	**Plasma**	-	-
Retinol	LC-MS/MS	-	-
25-OH-vitamin D	LC-MS/MS	-	-
α-tocopherol	LC-MS/MS	-	-
Phylloquinone	LC-MS/MS	-	-
Lipids	**Serum**	**-**	**Serum**
HDL-C	Direct, enzymatic inhibition[3]	-	Photometry[2]
LDL-C	Martin/Hopkins equation	-	Photometry[2]
Total cholesterol	Enzymatic method[3]	-	-
Triglycerides	Enzymatic method[3]	-	Photometry[2]
Ketones			
Acetoacetate	-	-	GC-MS/MS
ß-hydroxybutyrate	-	-	GC-MS/MS

Biomarker	Paper I	Paper II	Paper III
Acylcarnitines	-	-	**Serum**
Free carnitine			LC-MS/MS
Short-chain acylcarnitines			
Acetylcarnitine (C2)	-	-	LC-MS/MS
Propionylcarnitine (C3)	-	-	LC-MS/MS
Butyrylcarnitine (C4)	-	-	LC-MS/MS
Isovalerylcarnitine (iC5)	-	-	LC-MS/MS
Glutarylcarnitine (C5-DC)			LC-MS/MS
Medium-chain acylcarnitines			
Hexanoylcarnitine (C6)	-	-	LC-MS/MS
Octanoylcarnitine (C8)	-	-	LC-MS/MS
Decanoylcarnitine (C10)	-	-	LC-MS/MS
Dodecanoylcarnitine (C12)	-	-	LC-MS/MS

[1]All metabolites were quantified at Bevital AS (https://bevital.no/) unless otherwise indicated. [2]Quantified at the Department of Medical Biochemistry and Pharmacology at Haukeland University Hospital. [3]Quantified at the Department of Clinical Chemistry, Oslo University Hospital, Ullevål. **Abbreviations:** DC, Dicarboxylic; FMN, Flavin mononucleotide; GC-MS/MS, Gas chromatography-tandem mass spectrometry; HDL-C, High-density lipoprotein cholesterol; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LDL-C, Low-density lipoprotein cholesterol; NAM, Nicotinamide; MMA, methyl-malonic acid; mNAM, N1´-methylnicotinamide; PA, 4´-pyridoxic acid; PLP, Pyridoxal-5´-phosphate; TMP, thiamine monophosphate

4.2.1 Paper I

Serum samples of total cholesterol, HDL cholesterol, glucose, and triglycerides were analyzed within 7 days at the Department of Clinical Chemistry, Oslo University Hospital, Ullevål, with reagents from Boehringer Mannheim (Roche) as adapted to a Hitachi 911 analyzer. Cholesterol and triglycerides were measured by enzymatic methods, while HDL cholesterol was measured by a direct, enzymatic inhibition method. LDL cholesterol was calculated using the Martin/Hopkins equation as described by Martin et al. (111). Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. Analyses of amino acids, one-carbon metabolites, and B-vitamin biomarkers were conducted at Bevital AS (Bergen, Norway, http://bevital.no/). All amino acids, including the one-carbon metabolites cystathionine, cysteine, glycine, homocysteine, methionine, and serine, in addition to MMA, were measured in plasma using gas chromatography-tandem mass spectrometry (GC-MS/MS) (112). Plasma betaine, choline, and dimethylglycine, as well as the B-vitamin biomarkers thiamine, TMP, riboflavin, FMN, nicotinamide, N1´-methylnicotinamide (mNAM), pyridoxal, PLP, and 4´-pyridoxic acid were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (113, 114). Plasma folate and cobalamin were measured by a microbiological assay (115, 116), while the lipid-soluble vitamins retinol, 25-hydroxyvitamin D, α-tocopherol, and phylloquinone were measured in plasma using LC-MS/MS (112).

4.2.2 Paper II

All metabolites, except for cobalamin and folate, were analyzed at Bevital AS (Bergen, Norway) in samples that had been frozen and stored at -80ºC. The amino acids and one-carbon metabolites were analyzed in serum at Bevital AS (Bergen, Norway). Alanine, asparagine, aspartate, cystathionine, cysteine, glutamate, glutamine, glycine, histidine, total homocysteine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, sarcosine, serine, threonine, tryptophan, tyrosine, and valine were analyzed using GS-MS/MS, while arginine, betaine, choline, dimethylglycine, and methionine sulfoxide were analyzed using LC-MS/MS. FMN, mNAM, nicotinamide, pyridoxal,

PLP, 4′-pyridoxic acid, riboflavin, thiamine, and TMP were analyzed using LC-MS/MS, while MMA was analyzed using GC-MS/MS. Serum cobalamin and total serum folate (i.e. the sum of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and pteroylglutamic acid) at the Department of Medical Biochemistry and Pharmacology at Haukeland University Hospital (Bergen, Norway, certified NS-EN ISO 15189:2012) using immunoassay, in samples that had been chilled to +4ºC and stored for up to 24 hours before analysis.

4.2.3 Paper III

Serum HDL cholesterol, LDL cholesterol, and triglycerides were analyzed at the Department of Medical Biochemistry and Pharmacology at Haukeland University Hospital (Bergen, Norway) using photometry in samples that had been chilled to +4ºC and stored for up to 24 hours before analysis. Free carnitine, acylcarnitines, and ketones were analyzed in serum samples frozen to -80ºC until analysis. Free carnitine and all acylcarnitines were analyzed using LC-MS/MS, while acetoacetate and βHB were analyzed using GC-MS/MS at Bevital AS (Bergen, Norway).

4.3 Statistical analyses

The statistical analyses in the included papers were performed using R version 4.1.3 (the R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org/) and the packages within the *tidyverse* (117)*, irrICC,* and *emmeans*.

4.3.1 Paper I

For descriptive statistics, continuous variables were reported as geometric mean (gMean [95% prediction interval, PI]) and categorical variables as counts (percentages). The 95% PI provides the limits of the interval defined by $(gMean \div gSD^2, gMean \times gSD^2)$. The marginal gMean (95% geometric confidence interval [gCI]) metabolite concentration was estimated for each time category from a linear regression model adjusted for sex, age group, and BMI and presented visually as a

function of time since the last meal. The 95% gCI is constructed as the interval defined by $(gMean \div gSE^2, gMean \times gSE^2)$. Unadjusted gMean metabolite concentrations with 95% gCI at each timepoint were also plotted as a function of time since the last meal for the two age cohorts and for males and females separately. To explore the potential effects of sex and age, product terms for time*sex and time*age groups were included in the model.

4.3.2 Papers II and III

In **Paper II**, descriptive statistics were presented as gMeans (gSD) for continuous variables and counts (percentages) for categorical variables and supplemented with ranges (min-max). In both **Papers II** and **III**, all metabolite concentrations were log-transformed before statistical analysis and described using the back-transformed gMean and gSD as recommended (118, 119). Inferential statistics were accompanied by 95% gCI as a measure of uncertainty. The main objective was presented visually by plotting the raw metabolite concentrations as a function of time with the mean time-course indicated by superimposing the gMean concentrations (95% gCI) on top of the individual data. Relative changes in metabolite concentrations were calculated for each individual, with each pre-breakfast blood sample utilized as an individual reference value. These individual percentage changes were subsequently combined to calculate the gMean percentage change across the study cohort and presented visually.

4.4 Ethics

Paper I

The HUSK study was carried out in accordance with the Declaration of Helsinki. It was approved by the Regional Committee for Medical and Health Research Ethics (REK, REK No. 2009/825) and the Norwegian Data Inspectorate. All participants provided written informed consent. The analyses presented in the paper were approved by REK (REK No. 184165).

Papers II and III

The PoMet study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by REK (REK No. 236654). Written informed consent was obtained from all subjects. Participants received the consent form by email before the study visit to allow adequate time to read and understand the protocol and to familiarize themselves with the risks, burdens, and benefits of participation in the study. In addition, one of the researchers went through the consent form orally with the participants, and participants were explicitly allowed to ask additional questions before signing the consent form. Participants who communicated great discomfort orally or by body language (syncope, etc.) during the study visit were excluded from the study. Participants were also excluded if there were difficulties with blood sampling from the venous catheter.

5. Summary of main results

In this section, a summary of the main results will be presented across metabolite groups.

5.1 Amino acids

In the middle-aged and elderly adults in **Paper I**, we observed a consistent pattern for most plasma amino acids the first 7 hours after a meal (**Paper I**, **Figure 3**); the levels were highest in samples collected within the first 2 hours after a meal, and lowest in samples collected at 5-7 hours after a meal. This pattern was evident for plasma alanine, arginine, asparagine, aspartic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine. The largest relative differences between the highest and the lowest values were found for alanine (27%), isoleucine (25%), and proline (25%). Similar findings were observed in the younger adults in **Paper II**, in which these amino acids in serum, except for aspartic acid, peaked within the first 3 hours after the standardized breakfast meal and thereafter decreased (**Paper II**, **Figure 4**). The largest relative increases from baseline values were found for serum proline (56% increase), alanine (42%), tyrosine (29%), and isoleucine (28%) (**Paper II**, **Table 4**). Furthermore, the concentrations of the serum BCAAs increased from 6 and 8 hours to 24 hours after the meal, while serum proline decreased from 6 to 24 hours after the meal (**Paper II**, **Figure 4**). In the young, middle-aged, and elderly groups, male participants tended to have higher absolute concentrations of the amino acids; however, the patterns in amino acid concentrations after the meal were comparable between sexes.

5.2 One-carbon metabolites

In the middle-aged and elderly adults in **Paper I**, the concentrations of plasma betaine, choline, cystathionine, dimethylglycine, glycine, methionine, and serine were highest in samples collected the first 2 hours after a meal, while the lowest concentrations were

observed in samples collected at 5-7 hours after a meal (**Paper I**, **Figure 5**). The largest relative differences between the lowest and the highest values were found for methionine (29%) and cystathionine (26%). Similar results were also found in the young adults in **Paper II**, where these one-carbon metabolites in serum, in addition to sarcosine, increased and peaked within the first 3 hours after the meal (**Paper II, Figure 5**). As with the middle-aged and elderly adults, the largest relative increases were also found for cystathionine (38%) and methionine (29%) in the young cohort. The concentrations of these serum metabolites were thereafter relatively stable until 24 hours, except for cystathionine, which decreased until 12 hours (**Paper II**, **Table 4**). Notably, a distinct but similar pattern was observed for homocysteine and cysteine in all age groups; these one-carbon metabolites were lowest shortly after dietary intake, with increasing concentrations observed with increasing time since the last meal (**Figure 5** in **Papers I** and **II**). The observed patterns were largely consistent in males and females and across the age cohorts. However, for serum methionine sulfoxide, which was only explored in the younger adults, we observed that females had a higher peak in concentrations than males (+88% at 90 hours after the meal for females; +67% at 2 hours for males) (**Paper II, Table 5**).

5.3 Biomarkers for B-vitamin status

The biomarkers for B-vitamin status are presented in **Figure 7** in **Paper I** and **Figure 6** in **Paper II**. Both in the young adults (**Paper II**) and in the middle-aged and elderly adults (**Paper I)**, the concentrations of thiamine were highest shortly after dietary intake (+24% increase from baseline values in **Paper II**) and thereafter decreased with increasing time since the meal. A similar pattern was also found for TMP in all age groups; however, the concentrations fluctuated in the first hours after the meal in the young group before decreasing. Opposite patterns were found for riboflavin and FMN, where concentrations tended to be lowest after dietary intake and thereafter increase with increasing time since the last meal in all cohorts (+90% and +34% increase at 24 hours for FMN and riboflavin in **Paper II**, respectively). Notably, we observed different patterns for folate and cobalamin between the young and the middle-aged and

elderly adults; in the young group (**Paper II, Table 4**), we observed no considerable changes the first hours after the meal, except for a small peak in serum folate concentrations. Thereafter, serum cobalamin and folate concentrations steadily increased from 3 hours, peaking at 24 hours after the meal (+14% increase for cobalamin and +57% increase for folate). In the middle-aged and elderly groups (**Paper I, Table 2**), no clear patterns were observed for either plasma cobalamin or folate.

The results were largely similar between the sexes, except for the change in serum concentrations of mNAM and nicotinamide in the young adults (**Paper II**, **Table 5**). Concentrations of both mNAM and nicotinamide slightly increased the first hour after the meal in females and thereafter tended to decrease and remained lowered until 12 hours after the meal. In males, however, these metabolites increased after the meal and thereafter fluctuated but remained elevated or at baseline levels the first 8 hours after the meal before decreasing. However, these sex-based differences were not found for these plasma metabolites in **Paper I**.

5.4 Biomarkers for lipid-soluble vitamin status

The lipid-soluble vitamin biomarkers were explored only in the middle-aged and elderly adults and presented in **Paper I**, **Figure 6**. We observed no clear patterns for plasma retinol, 25-hydroxyvitamin D, or α-tocopherol. For plasma phylloquinone, the concentrations were highest in samples collected within the first hour after dietary intake, and thereafter, concentrations decreased with increasing time since the last meal, with lowest values in samples collected 6-7 hours after dietary intake. The observations for phylloquinone were observed in both age cohorts and in both sexes.

5.5 Lipids

The concentrations of serum lipids were explored both in the middle-aged and elderly adults and in young adults, and we observed slight differences between the two cohorts. In the young adults (**Paper III, Figure 1**), serum triglyceride concentrations increased

and peaked at 3 hours after the standardized meal (+28% increase from baseline values). Thereafter, concentrations decreased until 10 hours (-17% from baseline values) before increasing until 24 hours after the meal. However, in the middle-aged and elderly adults (**Paper I, Figure 4**), serum triglyceride concentrations were highest in samples collected 0-1 hours after dietary intake. Thereafter, concentrations decreased and reached the lowest values in samples collected 6-7 hours after dietary intake, with a mean difference of 15% between the highest and the lowest values.

In the young adults (**Paper III, Figure 1**), we observed that serum HDL- and LDL cholesterol concentrations slightly decreased shortly after the meal (-4.1% and -4.3% decrease from baseline values, respectively), followed by an increase peaking at 12 hours (HDL cholesterol; +6.0% increase from baseline values) and 24 hours (LDL cholesterol; +6.9% increase). In the middle-aged and elderly adults (**Paper I, Figure 4**), we also observed that concentrations of these serum lipids were lowest in samples collected 0-1 hours after a meal. However, while LDL cholesterol increased with increasing time since dietary intake and the highest values were observed in samples collected at 6-7 hours, the highest HDL cholesterol levels were observed in samples collected at 4-5 hours, however, with considerable uncertainty at the latest time points.

5.6 Ketones

Serum concentrations of the ketones acetoacetate and ßHB were explored only in the young adults and presented in **Paper III, Figure 2.** We observed that concentrations decreased shortly after the meal; acetoacetate reached its lowest levels at 15 minutes (-36% decrease), while ßHB reached its lowest levels at 90 minutes (-63% decrease). Starting 3 hours after the meal, concentrations steadily increased and reached the highest values at 24 hours, with an average increase of +433% for acetoacetate and +633% for ßHB compared to baseline levels. Concentration patterns were similar for both sexes (**Paper III, Table 2**).

5.7 Acylcarnitines

The concentrations of serum acylcarnitines were explored only in the young adults and presented in **Paper III, Figure 3.** We observed that acetylcarnitine and the medium-chain acylcarnitines decreased shortly after the meal, reaching their lowest values the first hours after the meal, with decreases ranging from −40% (acetylcarnitine and hexanoylcarnitine) to −70% (octanoylcarnitine). Subsequently, concentrations steadily increased, reaching the highest values at 24 hours, with increases ranging from +63% (acetylcarnitine) to +120% (dodecanoylcarnitine). Conversely, free carnitine and the short-chain acylcarnitines propionylcarnitine and isovalerylcarnitine increased and peaked 2 hours after the meal (17%, 19% and 15% increase, respectively). Concentrations thereafter decreased and remained lowered until 12 hours before returning to baseline values at 24 hours. Female participants tended to have lower concentrations of free carnitine and acylcarnitines compared to male participants. However, we observed no notable differences between sexes in the relative changes after the meal (**Paper III, Table 2**).

6. Discussion

The focus of this thesis was to explore biomarker concentrations in the hours following a meal and during the adaptation to the fasting state. Overall, we observed clear patterns in the concentrations of several biomarkers, both in middle-aged and elderly adults and in young adults. Further, these patterns were not confined to the immediate period after dietary intake but persisted for several hours after dietary intake. The subsequent section will delve into the methodological aspects of the two distinct projects employed in this thesis. Following that, a discussion of the main findings will be provided before the implications of our findings will be discussed.

6.1 Methodological considerations

6.1.1 Study designs

In this thesis, we utilized cross-sectional biomarker data from the observational HUSK study for **Paper I** and time series biomarker data from the interventional PoMet study for **Papers II** and **III**, which have important implications for the interpretation of the findings. As biomarkers were measured only once for each participant in HUSK, the biomarker concentrations at each timepoint represent the gMean value across all participants with a similar self-reported duration of time since their last meal. Since biomarker concentrations may have remained stable within individuals during the hours following a meal, yet varied between individuals, the observed patterns in **Paper I** only reflect group-level patterns. Therefore, the patterns observed should be interpreted within the context of group-level trends and cannot be extrapolated to hold true at the individual level.

Conversely, in the PoMet study, biomarkers were measured at a total of 14 time points during a 24-hour period, which allowed us to detect biomarker changes after the meal within each individual. When patterns are consistent between individuals, it strengthens the theory that these observed patterns are not merely a result of chance but

are more likely to signify a causal relationship. Furthermore, it has been stated that it is rare to find time series metabolomics data with more than 3-10 time points (120). The 14 sampling time points, particularly frequent the first 2 hours after the meal, serve as a notable strength as it allowed us to capture even small fluctuations in biomarker concentrations.

Instructions before the study visit

While no preparatory instructions were given to the participants in HUSK before the baseline visit, the participants in the PoMet study received instructions on physical activity, the use of dietary supplements, alcohol consumption, and the use of nicotine-containing products before the study visit. Additionally, they were instructed to consume a semi-standardized meal 12 hours before attending the study visit, as recommended (95). These instructions were given to reduce interindividual differences in the metabolome. However, these instructions also reduce the external validity of the results and the generalizability to a real-world setting where no such preparatory instructions before blood sampling are provided.

The meal before blood sampling

In HUSK, specific details regarding the meal consumed before blood sampling are unknown. The meal may have been breakfast, lunch, dinner, or a snack, meaning that the meal composition may have varied considerably. Furthermore, no information was available regarding whether this meal was the participants' first, second, or third meal of the day. However, there is no reason to believe there were systematic differences in the meals consumed across the time categories. Thus, the absence of a standardized meal is likely to have introduced variability to the results rather than bias. Also, the lack of a standardized meal increases the study's external validity by reflecting a real-world scenario where participants or patients do not receive preparatory instructions on meal choices before blood sampling.

Conversely, in the PoMet study, participants were provided a standardized meal before blood sampling to minimize potential variations in metabolite concentrations

that could arise from participants consuming different meals. To ensure the same nutrient intake for all participants, the same meal was given to all participants, regardless of their sex or body mass composition. However, providing the same meal to all participants could have introduced variability to the results, as we observed large variations in body size and composition and resting metabolic rate between participants. Thus, the meal may have been digested, and the nutrients metabolized at different rates between the participants (121). Furthermore, while efforts were made to compose the breakfast to resemble a typical Norwegian breakfast in terms of food items and macronutrient composition, the findings from the PoMet study may not necessarily apply to other breakfast meals, potentially limiting the generalizability of the results.

6.1.2 Bias

Bias is a major threat to the internal validity of a study, which is the characteristic of a study to produce valid results. Bias encompasses all factors that lead to a systematic deviation between the observations from a study and the actual truth and can occur at all stages of the research process. Many different sources of bias have been identified and can be broadly categorized into selection bias and information bias (122).

Selection bias

Selection bias refers to systematic differences between those who participate in a study and the source population, and is an inherent challenge when recruiting volunteers for research (122). The source population in the PoMet study comprised healthy, young individuals with BMIs between 22-27 kg/m^2, and we applied strict inclusion- and exclusion criteria for participation that resulted in a relatively homogenous group of participants. However, as we did not try to recruit a representative sample, 35 out of the 36 participants initially included in the study were of Caucasian ethnicity. This ethnic homogeneity could potentially introduce selection bias, as there may be differences in the blood metabolome between ethnicities (123, 124). It has also been shown that people willing to participate in research tend to be more health-conscious than those who do not. This phenomenon is commonly referred to as the *healthy volunteer effect* (125). Furthermore, it has been shown that invitees who decline to

participate in research are often overrepresented by individuals of lower socioeconomic status, poor self-reported health and an unhealthy lifestyle, receivers of disability benefits, and immigrants (126). Therefore, it is plausible to assume that both the PoMet and HUSK study participants were healthier overall than their respective source populations. However, whether this has introduced selection bias depends on whether the changes in biomarker concentrations after a meal differ between healthy and less healthy individuals. As the energy metabolism is well characterized, there is no reason to believe that the results would have been substantially different in less healthy populations.

Information bias

Information bias occurs during data collection if there is misclassification of binary or categorical variables or mismeasurement of continuous variables (127). It is a major threat to the internal validity of a study (122). In the present thesis, particular attention will be given to the measurement of the exposure and outcome variables, namely, time since the last meal and the various outcome biomarkers and metabolites.

Time since the last meal

In PoMet, we rigorously controlled the timing of the last meal in relation to blood sampling, particularly the first 12 hours after the meal. We recorded the completion time of the breakfast meal and, based on this information, determined the time of blood sampling. Moreover, we aimed to collect all blood samples within a ±2-minute window of the designated time point, which was achieved for most time points, with few exceptions. Notably, the 24-hour blood sample may be subject to greater uncertainty due to some participants returning to the study visit slightly earlier or later for convenience.

Conversely, in the HUSK study, information on time since the last meal relied on self-reporting. Unless participants adhered to strict eating schedules, it might have been challenging to recall the exact timing of their dietary intake earlier in the day, potentially introducing recall bias. Moreover, it is reasonable to assume that participants who had consumed their last meal several hours prior were more

susceptible to recall bias, as it can be more challenging to accurately remember the exact timing of meals consumed several hours ago compared to more recent meals consumed just before the study visit (128). However, since time was categorized into hourly intervals, minor errors in the recall of time since the last meal is unlikely to have a major impact on the results as most participants were probably included in the correct hourly category. Nonetheless, some participants may have been misclassified into the wrong category. Furthermore, categorizing time into hourly categories also presents a limitation. Time is inherently a continuous variable, and categorizing continuous variables is generally discouraged as it may lead to information loss and spurious results (129). However, given that participants were likely evenly distributed within each time category with regard to time since the last meal, the categorization of time is more likely to have increased variability in the results rather than introduce bias.

Outcome biomarkers and metabolites

Inadequate procedures in the preanalytical process can result in changes in numerous metabolites (68, 130). Consequently, several measures were undertaken in both the HUSK and the PoMet studies to ensure the collection of high-quality blood samples. First, the blood sampling in both projects was performed by qualified personnel, with strict adherence to protocols to ensure that procedures were carried out similarly for all blood samples. Second, as prolonged exposure of whole blood to room temperature may have a pronounced effect on the sample quality (65), stringent guidelines were followed to centrifuge, separate, and cool the samples within specified timeframes. Moreover, any samples not analyzed during the first days after blood sampling were stored at -80ºC, a widely accepted optimal storage condition. However, there were substantial differences in the storage duration of blood samples in the two projects. In the PoMet study, blood samples for analysis of all biomarkers and metabolites, except for serum lipids, folate, and cobalamin, were stored for up to 9 months without undergoing a freeze-thaw cycle before analysis. In contrast, in the HUSK study, the samples for analysis of amino acids, one-carbon metabolites, and vitamin biomarkers were stored from collection time in 1997-99 and underwent a few freeze-thaw cycles before being analyzed in 2010 and 2017. Storage at -80ºC over several years may affect

the stability of plasma samples (131). Any potential effect of the long-term storage or repeated freeze-thaw cycles will have impacted all the samples similarly, which may have introduced bias, as the absolute metabolite concentrations may have been systematically increased or decreased. However, as these factors were independent of time since the last meal, this potential bias is not likely to have impacted our overall observed patterns for the HUSK study. Additionally, it is important to highlight that most of the measured metabolites have been demonstrated to be relatively stable in EDTA plasma, irrespective of repeated freeze-thaw cycles, as tested by Bevital (https://bevital.no/stability-curves/).

The amino acids, one-carbon metabolites, acylcarnitines, and most vitamin biomarkers explored in this thesis were analyzed at Bevital AS using mass spectrometry-based methods to ensure accurate and precise quantification of the analytes (112). Another noteworthy strength is that the overlapping amino acids, one-carbon metabolites, and B-vitamin biomarkers in **Papers I** and **II**, except for folate and cobalamin, were analyzed at the same laboratory using consistent methods, enhancing the comparability between the papers. It should be noted that plasma was utilized in the middle-aged and elderly adults in the HUSK study, while serum was used in the young adults in the PoMet study. Previous studies have shown that serum samples often exhibit higher levels of most metabolites than plasma samples (70-74). However, this systematic difference is not believed to affect the observed changes in biomarker concentration. It is also worth mentioning that in **Paper I**, LDL cholesterol was calculated using the Martin/Hopkins equation instead of direct measurement as done in **Paper III**. The Martin/Hopkins equation has been shown to have the highest accuracy for estimating LDL cholesterol compared with other available equations. Nevertheless, direct measurement of LDL cholesterol is a more precise method than estimation (132).

6.1.3 Statistical methods and presentation of results

Given the explorative nature of the three papers, we opted for a descriptive approach with exploratory analyses when presenting the main results. Exploratory data analysis is a term coined by John W. Tukey to describe the act of *looking at data to see what it seems to say* (133). Rather than formal hypothesis testing to compare biomarker concentrations at specific time points, we emphasized visualizing the data when conveying our findings. This approach aligned with the primary aim of the studies, which was to explore the dynamics of biomarker concentrations, as opposed to determine at which timepoints biomarker concentrations deviated from each other. Additionally, formally testing time points against each other would introduce the challenge of *multiple comparisons*, which relates to the increased risk of obtaining false positive results when testing many associations (134). However, we did compare time points by reporting the relative difference between two time points (**Paper I**) and the relative changes from baseline values (**Papers II** and **III**). This was done to offer a more in-depth description of the observed patterns, such as describing the magnitude of the peak or highlighting biomarkers that appeared to have the largest changes after dietary intake. Moreover, merely observing patterns may not capture changes that could be of clinical importance.

When summarizing data, it is common to provide measures of central tendency, typically the mean, and the variability of the data, often represented as the SD. However, biological measurements, as utilized in the three papers, generally do not conform to a normal distribution, and tend to exhibit right-skewness, resembling a log-normal distribution (118). In log-normal distributions, most observations fall below the mean, and arithmetic measures to describe variability will often contain negative values. Since negative values are not possible for biological measurements, the log-normal variation is most appropriately characterized by the geometric mean (gMean) and the geometric standard deviation (gSD) (118). When describing the variation using the geometric measures, the gMean is divided and multiplied with the gSD, generating an asymmetrical interval containing all positive values. Therefore, we chose to represent baseline characteristics and metabolite concentrations using the gMean. In

Paper I, we opted to describe the distribution in baseline characteristics using 95% PI. It´s worth noting that gSD and 95% PI represent similar measures, as the 95% PI can be defined by $(gMean \div gSD^2, gMean \times gSD^2)$. However, 95% PI might be more intuitive for interpretation than gSD. In **Paper II**, we instead extended the descriptive statistics with ranges (minimum-maximum) and therefore reported gSD as is. The utilization of the range as a measure of distribution was deemed appropriate due to the homogeneity of the study population in PoMet.

In **Papers II** and **III**, we calculated the intraclass correlation coefficients (ICCs) for the various biomarkers and metabolites. The ICC is an index to assess the consistency of measurements taken from the same subjects and is indicated with a value between 0 and 1. It has been suggested that ICC values less than 0.5 indicate poor reliability, values between 0.5 and 0.75 indicate moderate reliability, and values between 0.75 and 0.9 indicate good reliability, while values greater than 0.9 indicate excellent reliability (135). In the context of the PoMet study, higher ICCs would imply more stable biomarker concentrations across the measurement time points. Various forms of ICC exist, and the different ICCs may produce different results when applied to the same data set. For **Papers II** and **III**, the ICCs were calculated based on a single-rater, absolute-agreement, two-way random-effects model as described by Koo and Li (135). The selection of the two-way random effects model was motivated by applying the same time points for the different participants, in contrast to the one-way random-effects model, which would have been the best choice if different time points were applied to the different participants. Furthermore, the time points were chosen as a random selection of several potential time points, and we planned to generalize our results to other time points with similar characteristics. The two-way mixed-effects model would have been a better choice if the time points chosen were the only time points of interest and the results could not be generalized to other time points. For the "definition" selection, we chose the absolute agreement as we were interested in the agreement between the time points, not the agreement between the participants at the different time points. If the latter was the case, the consistency definition would have been a better choice of definition. Finally, the single-rater type was chosen, although

we had 14 different time points, as the single time points were of interest rather than the mean of multiple time points. In the latter case, the mean of *k* raters would have been a better choice of type (135).

6.2 Discussion of main results across papers

Overall, we observed that several of the biomarkers explored in this thesis demonstrated clear patterns in the hours after dietary intake and during the adaptation to the fasting state. While these observed patterns may be attributed to time since the last meal, the observed patterns may also have occurred by chance or due to other factors such as time of day, seasonality, or meal composition. However, the probability of chance or other factors as the sole explanation is diminished by the remarkable consistency of these patterns between the HUSK study and the PoMet study, despite differences in study designs, study populations, and the number of blood samples collected in the two projects. The likelihood that the observed patterns are due to chance or other factors is further mitigated if underlying mechanisms that can provide a plausible explanation for the observed trends exist.

In the upcoming sections, an in-depth discussion of the main findings for the various biomarkers and metabolites explored both in the HUSK study and the PoMet study will be provided. A specific focus will be given to elucidating potential underlying mechanisms that could account for these observed patterns. Biomarkers and metabolites presented only in one of the papers (the lipid-soluble vitamins in **Paper I** and carnitine, acylcarnitines, and ketones in **Paper III**) are discussed therein.

6.2.1 Amino acids

Our findings for the amino acids, with peak concentrations after a meal, align with previous literature (95). Interestingly, we observed both in **Papers I** and **II** that proline and alanine seemed to elicit the largest postprandial response, as also observed by Badoud and colleagues (97). Additionally, we observed only small responses for glutamic acid in both **Papers I** and **II**, differing from the observed patterns of other

amino acids, despite glutamic acid being one of the most abundant amino acids in dietary protein (136). It has been suggested that glutamic acid is metabolized to various amino acids in the enterocyte during absorption, mainly to alanine, but also proline (136), offering a potential explanation for our observations. This finding underscores that the amino acid concentrations in blood after a meal cannot be used to quantify the amount of amino acids absorbed from a meal (96, 97). Furthermore, it highlights that consistent patterns were observed between projects, indicating that the meal consumed may be less important for the observed biomarker patterns.

In addition to the peak concentrations after the meal, we observed in **Paper II** that the BCAAs increased from 6 and 8 hours and reached the highest levels at 24 hours, which has also been observed by others during 36 hours of fasting (107, 108). It has been suggested that the increased concentrations during fasting are related to increased proteolysis in muscles (137), as the primary source of BCAAs in blood during fasting is protein degradation (138), predominantly derived from skeletal muscle (139).

In both **Papers I** and **II,** we observed that male participants tended to have, on average, consistently higher concentrations of most amino acids compared to female participants, which has also been observed previously (140, 141). However, we observed no noteworthy differences between males and females in concentration changes after dietary intake.

6.2.2 One-carbon metabolites

Both in **Papers I** and **II**, methionine and cystathionine appeared to be the one-carbon metabolites most responsive to dietary intake, with peak concentrations during the first hours after dietary intake. Methionine can be obtained from the diet, and unfortunately, we lack precise information on the methionine content in the meals before blood sampling for both papers. However, methionine is commonly found in foods regularly consumed in Western diets, such as meat, milk, cheese, nuts, beans, and whole grains (142), and was likely present in the meals before blood sampling in both studies. Thus, the methionine content in meals before blood sampling may explain the peak

concentrations after the meal. Methionine may be converted to cystathionine in the transsulfuration pathway (143), and it has previously been shown that cystathionine concentrations increase after the intake of methionine (144). In both **Papers I** and **II**, methionine concentrations peaked one hour before cystathionine, and this temporal pattern is consistent with methionine being released from protein in the food, followed by an increase in cystathionine. Moreover, the increased concentrations of cystathionine shortly after the meal may suggest an increased conversion of homocysteine to cystathionine through the transsulfuration pathway. This is supported by the observation of the lowest concentrations of both cysteine and homocysteine the first hours after the meal, both in **Papers I** and **II**, which has also been observed by others (145). The low concentrations of homocysteine and cysteine shortly after the meal may also be attributed to increased availability of choline and betaine that facilitate the remethylation of homocysteine to methionine in the homocysteine-methionine cycle.

In both **Papers I** and **II**, we observed peak concentrations of betaine, choline, dimethylglycine, glycine, sarcosine (only measured in **Paper II**), and serine in the first hours after dietary intake. These metabolites are involved in the choline oxidation pathway and are converted from choline and betaine. While the betaine and choline content in the meal before blood sampling in **Paper I** is unknown, the standardized breakfast meal in **Paper II** contained approximately 113 mg betaine and 25 mg choline as estimated by the United States Department of Agriculture (USDA) food database (142). Betaine and choline in the food may explain the increased concentrations of the metabolites in the choline oxidation pathway.

We observed both in **Papers I** and **II** that male participants tended to have, on average, consistently higher concentrations of most one-carbon metabolites, as expected (141). Furthermore, for methionine sulfoxide (**Paper II**), we observed higher peaks in females than in males. Methionine sulfoxide is formed by the oxidation of methionine and is suggested to be a marker of oxidative stress (146). While we have not identified any studies investigating the postprandial change in methionine

sulfoxide, higher concentrations of methionine sulfoxide among females have been previously reported (28, 147).

6.2.3 B-vitamin biomarkers

Our results for thiamine in **Papers I** and **II**, with peak concentrations shortly after dietary intake, is in line with previous findings (148). Peak concentrations of TMP after dietary intake was also observed in **Paper I** and to a smaller extent in **Paper II**. The peaks in both thiamine and TMP during the first 2 hours after a meal are likely attributable to thiamine content in the food, as both free thiamine and TMP enter the blood stream during absorption of thiamine (149). Our observation on FMN, with the lowest concentrations observed directly after dietary intake, is also comparable to previous findings (145, 148). As FMN serves as a cofactor in the electron transport chain, decreased concentrations may indicate increased utilization of this metabolite as a cofactor. In **Paper II,** we observed increased FMN- and riboflavin concentrations from 4 to 24 hours. To our knowledge, no studies have previously reported changes in FMN- or riboflavin concentrations in the fasting state. As the increase in both FMN- and riboflavin concentrations started around 4 hours after the meal, it is unlikely that the increase was due to the riboflavin content of the meal, and these findings require further investigations.

While no clear patterns for folate and cobalamin were found in **Paper I,** we observed increasing concentrations of these biomarkers from 3 hours after the meal in **Paper II**. It is possible that this potential change was not sufficiently captured in **Paper I**, where we only had data on the first 7 hours after dietary intake, and with greater uncertainty in the observations from 5-7 hours after dietary intake. Nonetheless, our finding for cobalamin in **Paper II** is not in line with previous findings. Orton and colleagues used cross-sectional data and reported that cobalamin concentrations decreased in males with increasing time since the last meal, up to 17 hours (150). The potential change in cobalamin concentrations after a meal warrants further investigation. For folate, a doubling in concentrations at 36 hours of fasting compared to shortly after a meal has previously been reported, similar to our findings. It has been

suggested that this increase may be explained by reduced excretion of folate in bile during fasting (151).

For most biomarkers of B-vitamin status, we did not observe any clear sex-based differences in the concentration patterns after dietary intake. Exceptions were for nicotinamide and mNAM in the young adults in **Paper II**; however, these sex-based differences were not noted in **Paper I**, suggesting a need for further investigations to elucidate potential variations.

6.2.4 Lipids

In both **Papers I** and **III**, triglyceride concentrations peaked after dietary intake. However, the highest concentrations were observed at 0-1 hour in the middle-aged and elderly adults in **Paper I** and at 3 hours in the young adults in **Paper III**. This observed difference might be age-related, as it has been demonstrated that there are age-related differences in postprandial triglyceride concentrations (152-154). Nonetheless, the peak in triglyceride concentrations during the first hour after dietary intake in the middle-aged and elderly adults differs from established knowledge on lipid metabolism, where triglyceride concentrations usually peak 3-5 hours after a meal (89). This observation might be explained by the participants included in **Paper I** were not asked to fast before their last reported meal, which is common in experimental studies, such as in the PoMet study in **Paper III**. Thus, the second meal effect may explain why we observed a different pattern in **Paper I** than in **Paper III** and the general literature (92, 93). Comparable to our findings, Mikkelsen and colleagues (155) used cross-sectional data from the Tromsø Study and observed that triglyceride concentrations were highest at 1 - 4 hours after a meal in individuals aged > 40 years.

In both **Papers I** and **III**, we observed the lowest concentrations of HDL- and LDL cholesterol shortly after dietary intake, which aligns with previous findings reported in the literature (153, 156-158). Langsted and colleagues suggested that the observed drop in concentrations could be caused by a hemodilution effect from fluid intake in relation to the meal (158); however, it has been argued that mechanisms other than hemodilution must be involved (159, 160). The observed concentration drop in

HDL cholesterol may be explained by augmented cholesterol ester transfer from HDL particles to very-low-density lipoproteins (VLDLs) and chylomicrons, facilitated by cholesteryl ester transfer protein (CETP) and LPL. This phenomenon is suggested to arise due to an influx of triglyceride−rich lipoproteins from the intestine (156, 157), which stimulates the activity of CETP and LPL. Additionally, the LDL cholesterol concentrations may be decreased due to enhanced triglyceride hydrolysis in chylomicrons catalyzed by LPL after the meal, as this process may inhibit the formation of LDL particles from VLDLs because VLDLs and chylomicrons compete for LPL (153, 161).

6.2.5 The metabolic mechanisms underlying the observations across biomarker and metabolite classes

While the distinct biomarker and metabolite classes have been presented separately in the various articles and within this thesis, the different biomarkers and metabolites are metabolically interconnected within a comprehensive network, as illustrated in a simplified overview in **Figure 1**. When reviewing the results across the different cohorts, we observe a remarkable consistency in metabolite patterns. Furthermore, the observed metabolite patterns are biologically plausible and fit current knowledge regarding the metabolic needs in the postprandial and fasting states. Together, this strengthens the robustness of our observations.

For instance, glucose was presented in **Papers I** and **II** to validate the other findings. The glucose results indicated that glucose was the preferred energy fuel after dietary intake, as expected. As glucose concentrations stabilized, our observations in **Paper III** revealed a concurrent decrease in the concentrations of free carnitine and increases in the medium-chain acylcarnitines. These observations suggest an increased need for free carnitine inside the cell to form acylcarnitine to facilitate ß−oxidation of fatty acids to be used for energy. The coherence of these findings is further supported by the observed increases in the ketone levels in **Paper III**, which are products of acetyl-CoA generated through the ß−oxidation of fatty acids, in the same period. Moreover, we observed in **Paper III** that the concentration of propionylcarnitine and isovalerylcarnitine followed a similar pattern to the BCAAs, both the first hours after

the meal in **Papers I** and **II**, as well as during fasting in **Paper II**. These specific acylcarnitines are byproducts of the BCAAs, and their concentrations increase when BCAA metabolism accelerates (61), providing a plausible explanation for the observed correlation. The increased concentrations of the BCAAs during fasting indicate increased protein degradation from skeletal muscle and release from liver to be used for energy. In summary, the examination of findings across the metabolite classes, when viewed in the context of established knowledge on human energy metabolism, underscores the consistency and coherence of our observations, marking a considerable strength in the present project.

6.2.6 Inter- and intraindividual variations

It should be noted that the reported patterns in this thesis are patterns described at the group level. However, despite the relative homogeneity of the PoMet cohort, we observed interindividual variations in concentration patterns. These interindividual differences may be attributed to several factors, including, but not limited to, differences in body composition, previous dietary intake, or variations in hormone levels among participants. Moreover, there is reason to believe that there may be intraindividual variations in the biomarker and metabolite concentrations, for instance, during the different phases of the menstrual cycle among females. Thus, the patterns observed at the group level in the PoMet study may not hold true for all individuals in the cohort, nor in all situations. The unique biomarker and metabolite profile for each participant underscores the necessity for tailored approaches in healthcare.

6.3 Implications

When using biomarkers and metabolites in clinical care and epidemiological studies, prandial status is usually accounted for by distinguishing between non-fasting and fasting blood samples. This is commonly done by applying a cutoff at a specific time since the last meal or by exclusively utilizing fasting samples taken more than 8 hours since the last caloric intake. However, the findings in this thesis, as well as previously published data, demonstrate that the concentrations of several biomarkers and

metabolites change dynamically within both the fasting and non-fasting categories. These findings challenge the prevailing practice, which traditionally relies on the binary fasting/non-fasting classification and may have important implications for clinical care and epidemiological studies utilizing biomarker- and metabolite data.

6.3.1 Biomarkers in clinical care

In clinical care, biomarkers are often measured on a single occasion. An implicit assumption is that the concentration measured at a single time point is representative of the longer-term exposure. This is not a reasonable assumption, and time since food intake is a major factor that must be considered. One of the most common applications of biomarkers in clinical care is their use as diagnostic biomarkers. In such situations, time since the last meal of blood sampling could be of importance in determining whether a patient receives a diagnosis. For example, in **Paper II**, 6 participants (17%) crossed the established cutoff for folate deficiency at 10 nmol/L (162). Thus, in a clinical setting, these participants would have been classified as folate deficient if their blood sample was taken before or during the first hours after the meal but not at later time points. This raises the concern that the diagnosis and treatment of patients may vary depending on the time since the last meal of blood sampling.

Therefore, it may be important to evaluate the potential impact of time since the last meal at the time of blood sampling. This could be done by developing and applying correction factors to the measured concentrations to estimate the concentrations at a time point consistent with the cutoffs. Another option is establishing different diagnostic cutoff values for biomarker concentrations based on the timing of blood sample collection, potentially improving the sensitivity and specificity of diagnostic biomarkers in clinical settings. The sensitivity of a biomarker applies to its ability to correctly identify patients with the condition (true positives), while specificity denotes its ability to correctly identify patients without the condition (true negatives). An ideal biomarker would possess 100% sensitivity and specificity, meaning it would flawlessly discriminate between individuals with and without a condition. However, due to individual differences, biomarker levels often overlap between these groups in practice

(163). Consequently, the sensitivity and specificity of a test are intrinsically linked to the choice of diagnostic cut-off, as depicted in **Figure 6A**. For instance, if the diagnostic cut-off was set at the line "a" in **Figure 6A,** the biomarker measurement would correctly classify all true positives as having the condition, achieving 100% sensitivity. However, it would fail to accurately classify true negatives as not having the condition, resulting in poor specificity. Conversely, a diagnostic cut-off at line "b" in **Figure 6A** would correctly identify all true negatives as not having the condition (100% specificity) but fall short of correctly classifying true positives as having the condition, yielding poor sensitivity. Consequently, sensitivity and specificity are inversely related, and defining an acceptable rate of false negatives and false positives typically guides the selection of a diagnostic cut-off (77, 163).

An illustration of how different cutoff values for samples taken at different time points could improve the biomarker's sensitivity and specificity is illustrated in **Figure 6B.** Using homocysteine as an example, we observed that the concentrations were lowest shortly after a meal and increased thereafter. Assuming this concentration profile is similar in individuals with and without a disease, homocysteine measured in samples taken 1 hour after a meal will systematically be lower than samples taken 8 hours after a meal. Thus, if a single diagnostic cutoff is applied to homocysteine without considering the time since the last meal, its diagnostic performance may be compromised. For instance, if homocysteine exhibits good sensitivity and specificity in samples taken 1 hour after a meal, applying the same cutoff to samples taken 8 hours after a meal could result in poor specificity and a higher proportion of false positives, as depicted in **Figure 6B**. Thus, to optimize the diagnostic performance of biomarkers, considering different diagnostic cutoffs for samples taken at varied time points could be a strategy. For example, setting a distinct diagnostic cutoff for homocysteine, such as line "c" in **Figure 6B**, for samples taken 8 hours after a meal could be a strategic approach. This tailored approach acknowledges the dynamic nature of biomarker concentrations over time.

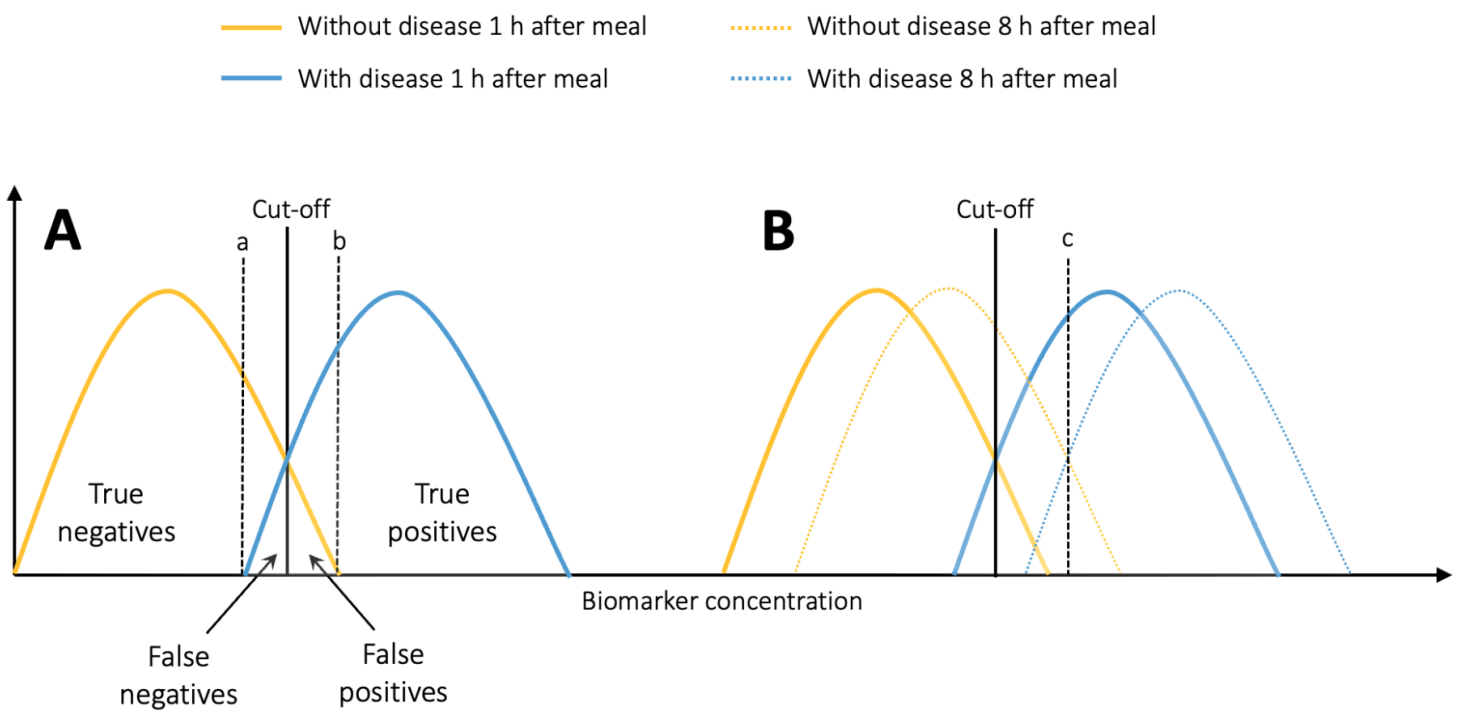

Figure 6. **A)** Diagram to illustrate that the sensitivity and specificity of a test depends on the diagnostic cutoff. If the diagnostic cut-off was set at the line "a", the test would have a 100% sensitivity but poor specificity. Conversely, a diagnostic cutoff at line "b" would have a 100% specificity but a poor sensitivity. **B)** Diagram to illustrate that if a biomarker changes in the hours after a meal, different diagnostic cutoffs could affect the biomarker's sensitivity and specificity. Applying a different diagnostic cutoff, for instance, at line "c" could improve the diagnostic performance of the biomarker.

Some biomarkers in clinical care are also used in contexts other than diagnostic biomarkers, for instance as monitoring biomarkers, to monitor the risk of a disease or the effect of treatment (7). In these situations, a biomarker is measured repeatedly. Thus, it may be essential that blood sampling is consistently conducted at the same time in relation to the time since the last meal for each measurement. For example, although the relative changes were modest, we observed that LDL cholesterol changed in the hours after dietary intake. If using LDL cholesterol as a monitoring biomarker to evaluate the effect of cholesterol-lowering drugs, the timing of blood sampling in relation to time since the last meal could be important: Changes in LDL cholesterol, which in reality are due to differences in sampling time points, may be wrongfully attributed to other factors such as the effect of cholesterol-lowering drugs.

6.3.2 Biomarkers and metabolites in epidemiological studies

The major role of epidemiological studies is to explore the association between an exposure and an outcome, often focusing on disease or mortality. In this context, metabolites are often modeled as the exposure, to investigate their role as potential biomarkers of disease. Furthermore, biomarkers may be modeled as confounders or outcomes of associations. When biomarkers are modeled as outcomes, they typically serve as a surrogate endpoint for the disease of interest. For example, if investigating the association between saturated fat intake and risk of cardiovascular disease, increased LDL cholesterol concentrations can be used as a surrogate endpoint for increased risk of cardiovascular events (7). If blood samples for biomarker or metabolite measurements are not drawn systematically with regard to time since the last meal between subjects in epidemiological studies, or if blood samples are categorized only into non-fasting and fasting blood samples, measurement error in the biomarker or metabolite may be introduced. The effect of this measurement error depends on the type of measurement error, and how the biomarker or metabolite is handled during data analysis (164).

Non-differential measurement error of an exposure occurs when the measurement error does not depend on another variable. If a biomarker or metabolite

is modeled as an exposure on a continuous scale, non-differential measurement error is, on average, expected to attenuate the observed associations due to *regression dilution bias* (127). However, as there is always a possibility that the measurement error may be unequally distributed by chance, attenuation of an association due to non-differential measurement error cannot automatically be assumed in single studies (165). Further, if the concentration of biomarker or metabolite is grouped into categories, such as quintiles, one cannot automatically assume an attenuation of the association, and the bias may be away from null for some of the exposure categories (166). If a biomarker or metabolite is modeled as a confounder, non-differential measurement error results in incomplete control for the confounder, and the effect estimate will be biased in the direction of the original confounding (127). Finally, if a biomarker or metabolite is modeled as the outcome of an association, non-differential measurement error in the outcome will decrease the precision of the estimate and thus require a larger sample size (167, 168).

Another problem arises if there is differential measurement error of the biomarker or metabolite. This occurs if the measurement error of the biomarker or metabolite depends on another variable, typically the outcome. This could occur if a biomarker or metabolite is measured at systematically different time points across individuals with different baseline risks, which is a particular concern in case-control studies (164). For instance, if blood sampling is conducted after an overnight fast for high-risk or diseased individuals but within a few hours after a meal for low-risk or healthy individuals, differential measurement error may be introduced. Differential measurement error could have different effects on the association: It could bias the associations towards the null, but also bias the association away from the null or even reverse the true association (*e.g.* a biomarker or metabolite may appear as protective when it is in fact increasing the risk). Thus, differential measurement error in biomarker or metabolite concentrations could lead to the wrong conclusion and completely invalidate a study, highlighting the importance of standardizing sampling procedures (164).

Therefore, to reduce measurement error in biomarkers and metabolites and increase the validity of epidemiological studies utilizing biomarker and metabolite data, we suggest that accounting for prandial status should be done by adjusting for the exact time since the last meal at the time of blood sampling. Using existing epidemiological data, researchers should consider adjusting for the exact number of hours since the last meal rather than distinguishing between non-fasting and fasting blood samples. In future epidemiological studies, researchers should strive to standardize the time of blood sampling as much as possible with regard to time since dietary intake.

7. Conclusions

Overall, the findings from this thesis indicate that the concentrations of several nutritional-related biomarkers and metabolites change in the hours after dietary intake. Considerable changes were found for nearly all amino acids and one-carbon metabolites, several vitamin biomarkers including thiamine, TMP, FMN, cobalamin, folate, and phylloquinone, the ketones, free carnitine, and short- and medium-chain acylcarnitines. Modest changes were also found for triglycerides, LDL- and HDL cholesterol in the hours after dietary intake. The concentration patterns were largely consistent in males and females and across different age-groups.

Our findings challenge the current, imprecise practice of distinguishing between non-fasting and fasting blood samples in clinical care and epidemiological research. To account for prandial status, the exact time since the last meal should be considered when interpreting blood samples. This could improve patient care when using biomarkers in the clinic and improve the validity of epidemiological studies utilizing biomarker and metabolite data.

8. Future perspectives

In the present thesis, we investigated how biomarker and metabolite concentrations change in the hours after habitual dietary intake. However, the observed changes in biomarker and metabolite concentrations should be confirmed in well-designed studies that investigate biomarker and metabolite concentrations in the hours following a habitual meal and meals with various nutrient compositions such as high-fat, low-fat, or low-carb meals. These studies should ideally include repeated blood samples within the same individuals. Further, as biomarker and metabolite concentrations may vary throughout the day, future research should explore patterns after meals at different times of the day to investigate the potential impact of circadian rhythms. The results from this thesis primarily pertain to a European population, and changes in biomarker and metabolite concentrations should be examined in populations of various ethnicities to determine if there are differences or common patterns across different ethnic groups. Finally, investigating biomarker patterns not only after the first meal of the day, but also after the second or third meal is valuable. One approach could involve mimicking real-world settings with several different meals during a day to examine the dynamics of biomarker concentrations throughout a 24-hour period. By addressing these areas, future research could contribute to a more comprehensive understanding of how biomarkers and metabolites change in the hours following dietary intake. This would provide the basis for improving the utilization of biomarkers and metabolites in clinical care and research settings.

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ORIGINAL CONTRIBUTION

Exploratory analyses on the effect of time since last meal on concentrations of amino acids, lipids, one-carbon metabolites, and vitamins in the Hordaland Health Study

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Abstract

Purpose Dietary intake may have pronounced effects on circulating biomarker concentrations. Therefore, the aim was to provide a descriptive overview of serum metabolite concentrations in relation to time since last meal, focusing on amino acids, lipids, one-carbon metabolites, and biomarkers of vitamin status.

Methods We used baseline data from the observational community-based Hordaland Health Study, including 2960 participants aged 46–49 years and 2874 participants aged 70–74 years. A single blood draw was taken from each participant, and time since last meal varied. Estimated marginal geometric mean metabolite concentrations were plotted as a function of time since last meal, up to 7 h, adjusted for age, sex, and BMI.

Results We observed a common pattern for nearly all amino acids and one-carbon metabolites with highest concentrations during the first 3 h after dietary intake. Homocysteine and cysteine were lowest the 1st hour after a meal, while no patterns were observed for glutamate and glutamic acid. The concentrations of phylloquinone and triglycerides were highest 1 h after dietary intake. Thiamine and thiamine monophosphate concentrations were highest, while flavin mononucleotide concentrations were lowest within the first 2 h after a meal. No clear patterns emerged for the other fat-soluble vitamins, blood lipids, or B-vitamin biomarkers.

Conclusion Our findings suggest that distinguishing between "fasting" and "non-fasting" blood samples may be inadequate, and a more granular approach is warranted. This may have implications for how to account for dietary intake when blood sampling in both clinical and research settings.

Keywords Biomarkers · Metabolites · Categorization · Fasting · Postprandial · Epidemiology

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Introduction

Blood metabolites are frequently used in epidemiological studies to assess the relationship with an incident disease or death. However, there are many sources of measurement error in metabolites when they are used in epidemiological studies. These measurement errors do not only encompass laboratory errors but may also arise from within-person variability. Variability in metabolite concentrations within subjects can be short term and caused by factors such as circadian rhythm and dietary intake, or long term caused by seasonal changes in diet or transient illness. As metabolite concentrations may fluctuate and change within subjects, a single measurement may inadequately capture the true etiologic exposure [1]. If a metabolite is modeled as an exposure, nondifferential measurement error can be expected to attenuate the risk association due to

regression dilution bias, and if it is modeled as a confounder, this may lead to residual confounding. Differential measurement error could bias the risk association in any direction and lead to incorrect conclusions from the study [1].

Dietary intake can affect blood metabolite concentrations, and marked metabolic and hormonal changes occur in the postprandial state [2–7]. Thus, the European Federation of Clinical Chemistry and Laboratory Medicine and the Latin America Confederation of Clinical Biochemistry have recommended that blood sampling should be conducted in subjects who have not eaten in the past 12 h [8]. Further, most epidemiological studies primarily utilize fasting blood samples taken at least 6 or 8 h since last dietary intake. However, healthy individuals in Western countries spend most of their awake time in the postprandial state (~ 18 h a day), and most people only enter the fasting state during an overnight sleep [9]. Thus, measuring circulating metabolites in fasting samples may not accurately measure the true exposure. For instance, recent findings suggest that a non-fasting lipid profile is superior to fasting for predicting cardiovascular risk, and several clinical guidelines and expert consensus statements now recommend non-fasting lipid testing for most clinical evaluations [10, 11]. Further, in epidemiological studies, collecting blood samples in participants 12 h fasting may be demanding for participants and is not always feasible.

As collecting blood samples in the hours following a meal is more convenient and may more accurately measure the true exposure, it is crucial to understand how specific metabolite concentrations may change during the postprandial state. If patterns in concentrations following a habitual meal could be identified, one could more precisely account for dietary intake and time since last meal in epidemiological studies. This could improve the internal and external validity of epidemiological studies utilizing metabolomic data. Thus, the main objective of the present study was to provide a descriptive overview of metabolite and biomarker concentrations in blood in the hours after dietary intake in community-dwelling middle-aged and elderly individuals from the Hordaland Health Study. This research question has previously been explored regarding homocysteine concentrations in the same cohort [12], but herein we aim to provide a more comprehensive overview and explore amino acids, lipids, metabolites related to the one-carbon metabolism, and biomarkers of vitamin status.

Methods

Study population

The study population included participants from the observational community-based Hordaland Health Study, where the baseline measurements were conducted during 1997–99 in Bergen, Norway. The cohort consisted of individuals aged 46–49 years (born 1950–51, referred to as the "middle-aged group," $n = 3089$), and individuals aged 70–74 years at baseline (born in 1925–27, referred to as the "elderly group," $n = 2969$) who were living in the city of Bergen or neighboring suburban municipalities. The data collection was conducted as a collaboration between the National Health Screening Service (now the Norwegian Institute of Public Health), the University of Bergen, and local health services. The study design and methodology have been described in more detail elsewhere [13].

A flowchart illustrating the inclusion and exclusion process of participants is shown in Fig. 1**.** In short, we excluded participants with missing information on time since last meal and participants with ≥ 7 h since last meal. This left us with a total of 5834 participants: 2960 participants in the middle-aged group and 2874 participants in the elderly group.

Data collection

The collection of demographics, clinical, and biochemical characteristics has been described in more detail elsewhere [13, 14]. In short, sociodemographic data were obtained by self-administered questionnaires. Participants underwent brief health examinations including measurements of height, weight, waist and hip circumferences, and blood pressure. Hypertension was defined as the use of medication for hypertension or the mean of at least two consecutive measurements of systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg. Participants were classified as diabetic based on self-reported questionnaires (previous or current diabetes), as well as a blood glucose ≥ 11.1 mmol/L within 2 h after dietary intake, or blood glucose ≥ 7 mmol/L more than 2 h after dietary intake. Information on nicotine exposure was collected by self-reported questionnaires and was verified by plasma cotinine (self-reported non-smokers with plasma cotinine levels > 85 nmol/L were classified as smokers) [15].

Blood sampling and biochemical analyses

Blood samples were collected at the first visit (between 8 a.m. and 6 p.m.), and the number of hours since last meal before blood sampling was recorded. Blood samples were only collected once from each participant, and all participants attended the first visit and provided blood samples with a different number of hours since last meal. There were a low number of participants who reported that their last meal was more than 7 h previously, so we only included participants with < 7 h after the meal. The time categories were given as follows: (1) 0– < 1 h, (2) 1– < 2 h, (3) 2– < 3 h,

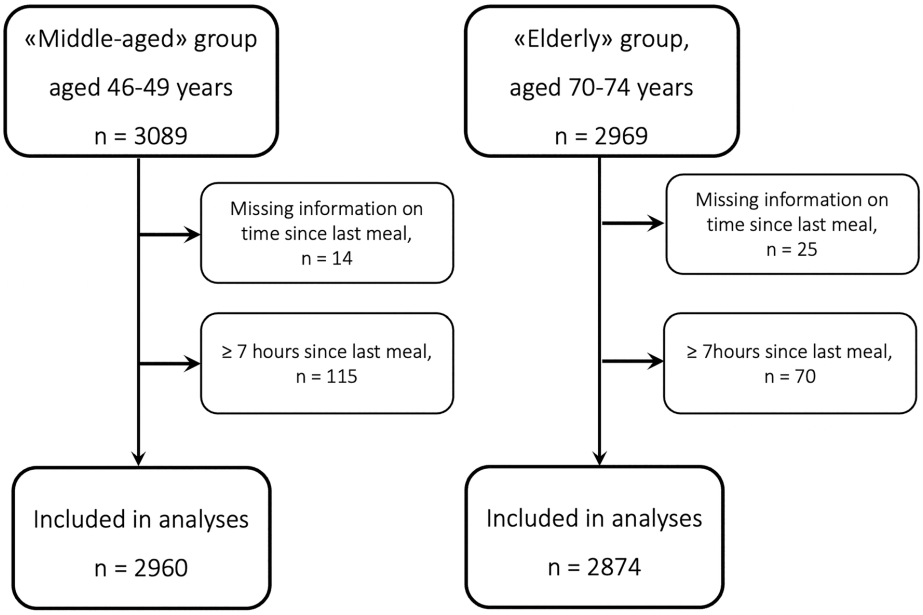

Fig. 1 Flowchart illustrating the inclusion and exclusion process of participants in the two age cohorts in the Hordaland Health Study 1997–1999

(4) 3–<4 h, (5) 4–<5 h, (6) 5–<6 h, and (7) 6–<7 h after a meal.

Serum was obtained by collecting blood into Vacutainer tubes with no additive. Blood was allowed to clot at room temperature for 30 min before isolation of the serum fraction. Plasma samples were collected into evacuated tubes containing EDTA, chilled at 4–5 °C within 15–30 min, and then centrifuged at 4000×*g* at 10 °C for 10 min within 1–3 h. Aliquots of serum and plasma were stored at − 80 °C until analysis. Serum samples of total cholesterol, high-density lipoprotein (HDL) cholesterol, glucose, and triglycerides were analyzed within 7 days at the Department of Clinical Chemistry, Oslo University Hospital, Ullevål, with reagents from Boehringer Mannheim (Roche) as adapted to a Hitachi 911 analyzer. Cholesterol and triglycerides were measured by enzymatic methods, while HDL cholesterol was measured by a direct, enzymatic inhibition method. Low-density lipoprotein (LDL) cholesterol was calculated using the Martin/Hopkins equation as described by Martin et al. [16]. This method has been shown to have better accuracy than the Friedewald equation for estimating LDL cholesterol and is the preferred method for estimating LDL cholesterol [17]. Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. Analyses of amino acids, one-carbon metabolites, and B-vitamin biomarkers were conducted at the Bevital A/S Laboratory, Bergen, Norway (http://bevital.no/). All amino acids, including the one-carbon metabolites cystathionine, cysteine, glycine, homocysteine, methionine, and serine, in addition to methylmalonic acid (MMA) were measured in plasma using gas chromatography–tandem mass spectrometry [18]. Plasma betaine, choline, and dimethylglycine, as well as the B-vitamin biomarkers thiamine, thiamine monophosphate (TMP), riboflavin, flavin mononucleotide (FMN), nicotinamide, methyl-nicotinamide, pyridoxal, pyridoxal-5´-phosphate (PLP), and 4-pyridoxic acid were measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [19, 20]. Plasma folate and cobalamin were measured by a microbiological assay [21, 22], while the lipid-soluble vitamins retinol, 25-hydroxyvitamin D, α-tocopherol, and phylloquinone were measured in plasma using LC–MS/MS [18]. More comprehensive information on the analytical platforms that were used to analyze the amino acids, one-carbon metabolites, and vitamin biomarkers is provided in Supplementary Table 1.

Statistical analyses and presentation of the results

All statistical analyses were performed using R, version 1.4.1717 (The R Foundation for Statistical Computing, Vienna, Austria), including the packages within the *tidyverse (tidyr, dplyr, broom,* and *ggplot2)* [23], *ggtext*, and *emmeans*. Continuous variables are reported as geometric means (95%

prediction intervals, PI) and categorical variables as counts (percentages). The marginal geometric mean (95% geometric CI) concentration was estimated for each time category from a linear regression model adjusted for sex, age group, and BMI and presented visually as a function of time since last meal. The *p*-value for time since last meal is presented on the figures. Unadjusted geometric mean metabolite concentrations with 95% geometric CIs at each timepoint were also plotted as a function of time since last meal for the two age cohorts and for males and females separately. To explore the potential effects of sex and age, product terms for time*sex, and time*age groups, were included in the model, and the *p*-values added to the figures.

Ethics

The Hordaland Health Study was carried out in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics (REK, REK No. 2009/825) and the Norwegian Data Inspectorate. All participants provided written informed consent. The analyses presented here were approved by REK (REK No. 184165).

Results

Characteristics of the study participants

A total of 5834 participants were included in the statistical analyses. The main characteristics of the study participants are presented in Table 1. The middle-aged cohort (aged 46–49 years, $n = 2960$) consisted of 42.5% male participants, while the elderly cohort (aged 70–74 years, $n = 2874$) consisted of 44.6% male participants. The average BMI was slightly lower in the middle-aged cohort (25.1 kg/m^2) compared to the elderly cohort (25.8 kg/m^2). A total of 279 participants (9.4%) had hypertension in the middle-aged cohort, of which 130 participants were classified as hypertensive based on the use of blood pressure medications, while 149 participants were classified based on blood pressure measurements. Further, 39 participants (1.3%) were classified as diabetic, of which 23 participants were previously diagnosed with diabetes, while 16 participants were classified as having diabetes based on blood glucose levels observed during initial study sampling. In the elderly cohort, 1023 participants (35.6%) had hypertension, of which 805 used blood pressure medications. Further, 252 participants (8.8%) were diabetic, of which 184 participants had an existing diagnosis of diabetes.

Metabolite concentrations as a function of time since last meal

The estimated marginal geometric mean metabolite concentrations in the seven different time categories are provided in Table 2. Results for the two age cohorts are presented in Supplementary Table 2, and results for males and females separately are presented in Supplementary Table 3. The number of missing values of the metabolite concentrations at each timepoint is provided in Supplementary Table 4 (total cohort) and Supplementary Table 5 (missing values for the two age groups, and males and females separately).

Glucose

The results for glucose concentrations as a function of time since last meal are presented in Fig. 2 and Table 2**.** As expected, we observed the highest concentrations of glucose during the 1st hour (5.99 mmol/L), with concentrations decreasing and reaching the lowest values at 4–5 h after the meal (4.99 mmol/L) and thereafter stabilizing. We observed no considerable differences in the two age groups (Supplementary Fig. 1) or between sexes (Supplementary Fig. 2).

Table 1 Main characteristics of the 5834 study participants in the Hordaland Health Study 1997–1999

	Middle-aged group (46–49 years), $n = 2960$	Elderly group (70–74 years), $n = 2874$
Age, years	47 (46, 49)	72 (70, 74)
Male, *n* (%)	1258 (42.5%)	1283 (44.6%)
Waist circumference, cm	84.7 (64.7, 111)	88.4 (67.7, 116)
Hip circumference, cm	101 (88.0, 115)	100 (87.0, 116)
Body mass index, kg/m^2	25.1 (18.9, 33.3)	25.8 (19.2, 34.6)
Current smokers, *n* (%)	1071 (36.2%)	513 (17.8%)
Former smokers, *n* (%)	782 (26.4%)	1086 (37.8%)
Diabetes mellitus (type 1 or 2), *n* (%)	39 (1.3%)	252 (8.8%)
Hypertension, *n* (%)	279 (9.4%)	1023 (35.6%)

Continuous variables are presented as geometric means (95% prediction interval) and categorical variables as counts (%)

Table 2 Estimated marginal geometric mean metabolite concentrations during the first 7 h after dietary intake in 5834 participants in the Hordaland Health Study 1997–1999

Hours since last meal	0–<1	1–<2	2–<3	3–<4	4–<5	5–<6	6–<7
***n* participants**[a]	537	1628	1533	1121	701	234	81
Serum glucose	5.99 (5.89, 6.09)	5.46 (5.41, 55.1)	5.28 (5.23, 5.33)	5.03 (4.97, 5.09)	4.99 (4.92, 5.06)	5.06 (4.94, 5.19)	5.01 (4.81, 5.23)
Amino acids							
Plasma alanine, µmol/L	404 (396, 411)	422 (417, 426)	393 (389, 397)	359 (355, 364)	347 (341, 352)	333 (324, 342)	332 (317, 349)
Plasma arginine, µmol/L	48.2 (47.2, 49.3)	50.5 (49.9, 51.2)	48.3 (47.7, 48.9)	44.9 (44.3, 45.6)	43.9 (43.1, 44.7)	41.6 (40.3, 43.0)	42.6 (40.3, 45.0)
Plasma asparagine, µmol/L	50.2 (49.3, 51.0)	50.7 (50.2, 51.2)	47.8 (47.3, 48.3)	44.7 (44.1, 45.2)	44.5 (43.8, 45.2)	43.2 (42.1, 44.4)	42.5 (40.6, 44.4)
Plasma aspartic acid, µmol/L	8.66 (8.47, 8.86)	8.93 (8.82, 9.05)	8.64 (8.52, 8.76)	8.15 (8.03, 8.28)	7.84 (7.69, 8.00)	7.80 (7.54, 8.07)	7.81 (7.37, 8.27)
Plasma glutamic acid, µmol/L	95.0 (92.1, 97.9)	93.7 (92.1, 95.3)	92.8 (91.2, 94.5)	90.0 (81.1, 91.9)	89.0 (86.7, 91.4)	89.9 (85.9, 94.0)	91.9 (85.0, 99.3)
Plasma glutamine, µmol/L	522 (514, 530)	527 (522, 531)	517 (513, 522)	506 (501, 512)	516 (510, 523)	512 (501, 523)	513 (494, 532)
Plasma histidine, µmol/L	82.5 (81.6, 83.4)	83.4 (82.8, 83.9)	80.8 (80.3, 81.4)	77.3 (76.7, 77.9)	77.9 (77.2, 78.7)	75.4 (74.2, 76.7)	74.8 (72.8, 76.9)
Plasma isoleucine, µmol/L	77.5 (75.7, 79.4)	77.9 (76.8, 78.9)	73.1 (72.1, 74.1)	66.5 (65.5, 67.6)	66.9 (65.5, 68.2)	65.3 (63.1, 67.7)	62.2 (58.6, 66.1)
Plasma leucine, µmol/L	139 (136, 142)	140 (138, 142)	132 (130, 134)	122 (120, 123)	122 (120, 124)	120 (116, 123)	116 (110, 123)
Plasma lysine, µmol/L	183 (180, 186)	191 (189, 193)	183 (181, 185)	171 (169, 173)	168 (166, 171)	160 (156, 164)	156 (149, 163)
Plasma phenylalanine, µmol/L	65.4 (64.5, 66.4)	66.5 (66.0, 67.1)	63.2 (62.7, 63.8)	58.6 (58.0, 59.2)	58.0 (57.2, 58.7)	56.1 (54.9, 57.3)	57.8 (55.7, 60.0)
Plasma proline, µmol/L	220 (215, 225)	226 (223, 229)	216 (214, 219)	198 (195, 201)	190 (186, 194)	181 (175, 187)	182 (172, 193)
Plasma threonine, µmol/L	127 (125, 130)	132 (130, 133)	126 (125, 128)	119 (118, 121)	119 (117, 121)	116 (112, 119)	116 (111, 123)
Plasma tryptophan, µmol/L	69.3 (68.1, 70.5)	71.7 (71.0, 72.4)	68.6 (67.9, 69.3)	63.4 (62.7, 64.2)	61.9 (61.0, 62.8)	60.0 (58.5, 61.6)	60.5 (57.9, 63.2)
Plasma tyrosine, µmol/L	67.8 (66.4, 69.2)	70.3 (69.5, 71.1)	66.9 (66.1, 67.7)	63.3 (62.4, 64.2)	60.7 (57.9, 61.8)	59.5 (57.8, 61.4)	57.9 (55.0, 61.0)
Plasma valine, µmol/L	263 (259, 267)	265 (263, 268)	259 (256, 261)	244 (241, 247)	245 (241, 248)	238 (233, 244)	232 (222, 242)
Lipids							
Serum total cholesterol, mmol/L	5.78 (5.69, 5.87)	5.84 (5.78, 5.89)	5.88 (5.83, 5.94)	5.93 (5.86, 5.99	5.92 (5.84, 6.00)	5.88 (5.75, 6.02)	5.96 (5.73, 6.20)
Serum LDL cholesterol, mmol/L	3.70 (3.62, 3.78)	3.74 (3.69, 3.78)	3.80 (3.75, 3.84)	3.84 (3.78, 3.89)	3.83 (3.76, 3.90)	3.81 (3.70, 3.93)	3.95 (3.74, 4.16)
Serum HDL cholesterol, mmol/L	1.25 (1.22, 1.28)	1.27 (1.26, 1.29)	1.26 (1.25, 1.28)	1.30 (1.28, 1.32)	1.30 (1.27, 1.32)	1.27 (1.23, 1.31)	1.25 (1.19, 1.32)
Serum triglycerides, mmol/L	1.64 (1.58, 1.70)	1.61 (1.58, 1.65)	1.57 (1.54, 1.61)	1.55 (1.51, 1.59)	1.48 (1.43, 1.53)	1.48 (1.40, 1.57)	1.43 (1.29, 1.58)

Table 2 (continued)

Hours since last meal	0–<1	1–<2	2–<3	3–<4	4–<5	5–<6	6–<7
One-carbon metabolites							
Plasma betaine, µmol/L	38.8 (37.9, 39.7)	39.6 (39.0, 40.1)	39.5 (39.0, 40.1)	37.5 (36.9, 38.1)	37.1 (36.4, 37.9)	34.9 (33.7, 36.1)	36.3 (34.2, 38.5)
Plasma choline, µmol/L	9.99 (9.81, 10.2)	10.1 (10.0, 10.2)	9.75 (9.65, 9.90)	9.58 (9.46, 9.70)	9.22 (9.07, 9.40)	8.76 (8.53, 9.0)	8.86 (8.46, 9.30)
Plasma cystathionine, µmol/L	0.22 (0.21, 0.23)	0.23 (0.23, 0.24)	0.24 (0.23, 0.24)	0.22 (0.21, 0.23)	0.22 (0.21, 0.23)	0.19 (0.18, 0.20)	0.19 (0.17, 0.21)
Plasma cysteine, µmol/L	303 (299, 307)	298 (296, 300)	302 (299, 304)	303 (300, 306)	308 (304, 311)	313 (307, 320)	316 (305, 327)
Plasma dimethylglycine, µmol/L	4.48 (4.38, 4.59)	4.60 (4.54, 4.66)	4.52 (4.46, 4.58)	4.45 (4.38, 4.52)	4.39 (4.30, 4.48)	4.24 (4.09, 4.38)	4.38 (4.14, 4.65)
Plasma glycine, µmol/L	250 (245, 256)	254 (251, 257)	251 (248, 254)	245 (242, 249)	239 (234, 243)	242 (234, 250)	236 (224, 250)
Plasma homocysteine, µmol/L	10.8 (10.5, 11.0)	10.9 (10.7, 11.0)	11.1 (10.9, 11.2)	11.0 (10.8, 11.1)	11.4 (11.2, 11.6)	11.6 (11.2, 12.1)	12.2 (11.5, 13.0)
Plasma methionine, µmol/L	24.8 (24.2, 25.4)	25.8 (25.4, 26.2)	23.8 (23.5, 24.2)	21.3 (21.0, 21.7)	20.9 (20.5, 21.4)	20.0 (19.2, 20.7)	20.2 (19.0, 21.6)
Plasma serine, µmol/L	117 (115, 119)	118 (117, 119)	114 (113, 115)	109 (108, 110)	109 (108, 111)	110 (107, 113)	108 (104, 113)
Lipid-soluble vitamins							
Plasma retinol, µmol/L	2.11 (2.07, 2.15)	2.16 (2.14, 2.19)	2.18 (2.15, 2.20)	2.19 (2.16, 2.22)	2.17 (2.14, 2.21)	2.14 (2.08, 2.20)	2.19 (2.08, 2.29)
Plasma 25-OH vitD, nmol/L	63.5 (61.9, 65.1)	64.6 (63.7, 65.6)	64.6 (63.7, 65.6)	65.5 (64.4, 66.7)	65.8 (64.3, 67.3)	63.5 (61.6, 66.0)	65.5 (61.3, 69.9)
Plasma α-tocopherol, µmol/L	36.2 (35.4, 37.0)	35.8 (35.4, 36.2)	36.0 (35.5, 36.4)	36.8 (36.3, 37.4)	36.3 (35.6, 37.0)	37.1 (35.9, 38.3)	36.7 (34.7, 38.7)
Plasma phylloquinone, nmol/L	1.71 (1.64, 1.80)	1.69 (1.64, 1.73)	1.62 (1.57, 1.66)	1.51 (1.47, 1.56)	1.53 (1.46, 1.58)	1.50 (1.39, 1.61)	1.28 (1.13, 1.45)
B-vitamin biomarkers							
Plasma thiamine, nmol/L	3.34 (3.16, 3.52)	3.42 (3.31, 3.53)	3.18 (3.08, 3.28)	3.03 (2.92, 3.15)	2.86 (2.72, 3.00)	2.65 (2.44, 2.87)	2.60 (2.34, 3.09)
Plasma TMP, nmol/L	7.52 (7.27, 7.79)	7.69 (7.54, 7.85)	7.23 (7.08, 7.38)	6.84 (6.68, 7.01)	6.90 (6.69, 7.11)	6.58 (6.24, 6.93)	6.34 (5.80, 6.93)
Plasma riboflavin, nmol/L	15.2 (14.2, 16.3)	15.0 (14.4, 15.6)	14.3 (13.7, 14.8)	14.2 (13.6, 14.9)	14.2 (13.4, 15.1)	14.4 (13.1, 16.0)	16.2 (13.7, 19.2)
Plasma FMN, nmol/L	12.6 (12.2, 13.1)	12.1 (11.8, 12.3)	12.4 (12.2, 12.7)	13.5 (13.2, 13.8)	14.4 (14.0, 14.8)	14.9 (14.2, 15.7)	15.2 (13.9, 16.6)
Plasma nicotinamide, nmol/L	367 (352, 382)	383 (375, 392)	388 (379, 397)	400 (388, 411)	374 (361, 388)	409 (385, 435)	401 (362, 445)
Plasma methylnicotinamide, nmol/L	85.8 (81.6, 90.2)	94.0 (91.3, 96.8)	87.6 (85.0, 90.3)	90.0 (86.9, 93.3)	85.2 (81.5, 89.0)	86.7 (80.3, 93.6)	87.3 (76.7, 99.3)
Plasma pyridoxal, nmol/L	14.1 (13.4, 14.8)	14.1 (13.7, 14.6)	13.2 (12.8, 13.6)	12.8 (12.4, 13.3)	12.6 (12.1, 13.2)	12.7 (11.7, 13.7)	13.2 (11.6, 15.0)
Plasma PLP, nmol/L	54.8 (52.2, 57.7)	56.3 (54.7, 57.9)	53.6 (52.0, 55.2)	51.8 (50.1, 53.7)	51.3 (49.1, 53.6)	51.1 (47.4, 55.1)	51.0 (44.9, 58.0)

Table 2 (continued)

Hours since last meal	0–<1	1–<2	2–<3	3–<4	4–<5	5–<6	6–<7
Plasma 4-pyridoxic acid, nmol/L	27.5 (25.9, 29.1)	27.6 (26.7, 28.5)	26.5 (25.6, 27.5)	27.2 (26.1, 28.3)	26.4 (25.1, 27.8)	25.8 (23.6, 28.2)	25.8 (22.2, 30.0)
Plasma folate, nmol/L	7.13 (6.82, 7.46)	7.03 (6.85, 7.22)	6.86 (6.68, 7.05)	7.00 (6.78, 7.23)	6.91 (6.64, 7.19)	6.99 (6.53, 7.48)	6.52 (5.80, 7.32)
Plasma cobalamin, pmol/L	352 (341, 3.64)	352 (346, 359)	351 (344, 357)	355 (347, 363)	352 (342, 362)	370 (353, 389)	344 (316, 374)
Plasma MMA, µmol/L	0.20 (0.19, 0.20)	0.20 (0.20, 0.20)	0.19 (0.19, 0.20)	0.19 (0.19, 0.19)	0.19 (0.18, 0.19)	0.18 (0.18, 0.19)	0.20 (0.18, 0.21)

All values are presented as estimated marginal geometric means (95% confidence intervals), adjusted for age cohort, sex, and body mass index

FMN flavin mononucleotide, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *MMA* methylmalonic acid, *PLP* pyridoxal 5'-phosphate, *TMP* thiamine monophosphate

[a]An overview of missing observations at each timepoint for each of the metabolites is found in Supplementary Table 4

Amino acids

Among the amino acids, we observed a common pattern for alanine, arginine, asparagine, aspartic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine (Fig. 3**,** Table 2**)**. The concentrations of these amino acids were highest 1–2 h after a meal, with the lowest concentrations observed at 5–7 h. The difference between the highest and the lowest values was ≥ 10% for all these amino acids, with the largest differences found for alanine (90 µmol/L, 27%), isoleucine (15.7 µmol/L, 25.2%), and proline (45 µmol/L, 24.9%). Findings were consistent in both age cohorts (Supplementary Fig. 3) and both sexes (Supplementary Fig. 4). No consistent patterns were observed for glutamic acid or glutamine.

Lipids

For the lipids **(**Fig. 4**,** Table 2**),** we observed that the concentrations of total cholesterol and LDL cholesterol in the total cohort were lowest in the first 2 h after a meal (5.78 and 3.70 mmol/L, respectively) and highest 6–7 h after a meal (5.96 and 3.95 mmol/L). The maximum mean difference between the lowest and the highest values was 0.18 mmol/L (3.1%) for total cholesterol and 0.25 mmol/L (6.8%) for LDL cholesterol. We observed some age differences, with concentrations being highest at 6–7 h after a meal in the middle-aged group, while in the elderly group, the concentrations were highest at 3–5 h after a meal (Supplementary Fig. 5). Further, HDL cholesterol concentrations were highest at 4–5 h after a meal (1.30 mmol/L) and lowest at 1–2 and 6–7 h after food intake (1.25 mmol/L). For the triglycerides, we observed the highest concentrations the first 2 h after a meal (1.64 mmol/L) and lower concentrations thereafter (2–7 h), with the lowest concentrations observed at 6–7 h after a meal (1.43 mmol/L), a difference of 0.21 mmol/L (14.7%). We observed no considerable sex or age differences for HDL or the triglycerides (Supplementary Fig. 5 and Supplementary Fig. 6).

One-carbon metabolites

For the one-carbon metabolites **(**Fig. 5**,** Table 2*),* we observed that the levels of homocysteine and cysteine were lowest during the 1st hours after a meal (10.8 µmol/L and 298 µmol/L, respectively), with concentrations peaking at 6–7 h (12.2 µmol/L and 316 µmol/L). For betaine, choline, cystathionine, dimethylglycine, glycine, methionine, and serine, we observed a pattern with the highest concentrations 1–2 h after a meal, and the lowest concentrations usually observed at 5–7 h after a meal. The relative difference between the highest and the lowest values was lowest for cysteine (18 µmol/L, 6.0%), glycine (18 µmol/L, 7.6%), and dimethylglycine (0.36 µmol/L, 8.5%) and highest for methionine (5.8 µmol/L, 29.0%) and cystathionine (0.05 µmol/L, 26.3%). We observed no noteworthy age or sex differences in the concentrations of any of the one-carbon metabolites as a function of time since last meal (Supplementary Figs. 7 and 8, respectively).

Lipid-soluble vitamins

Among the lipid-soluble vitamins **(**Fig. 6**,** Table 2**)**, no clear patterns were observed for retinol, 25-hydroxyvitamin D, or α-tocopherol. For phylloquinone, we observed peak concentrations in the 1st hour after a meal (1.71 nmol/L) and lower concentrations thereafter (2–7 h), with the lowest values observed at 6–7 h after a meal (1.28 nmol/L), giving a maximum mean difference of 0.43 nmol/L (33.6%). These observations were also observed in both age cohorts

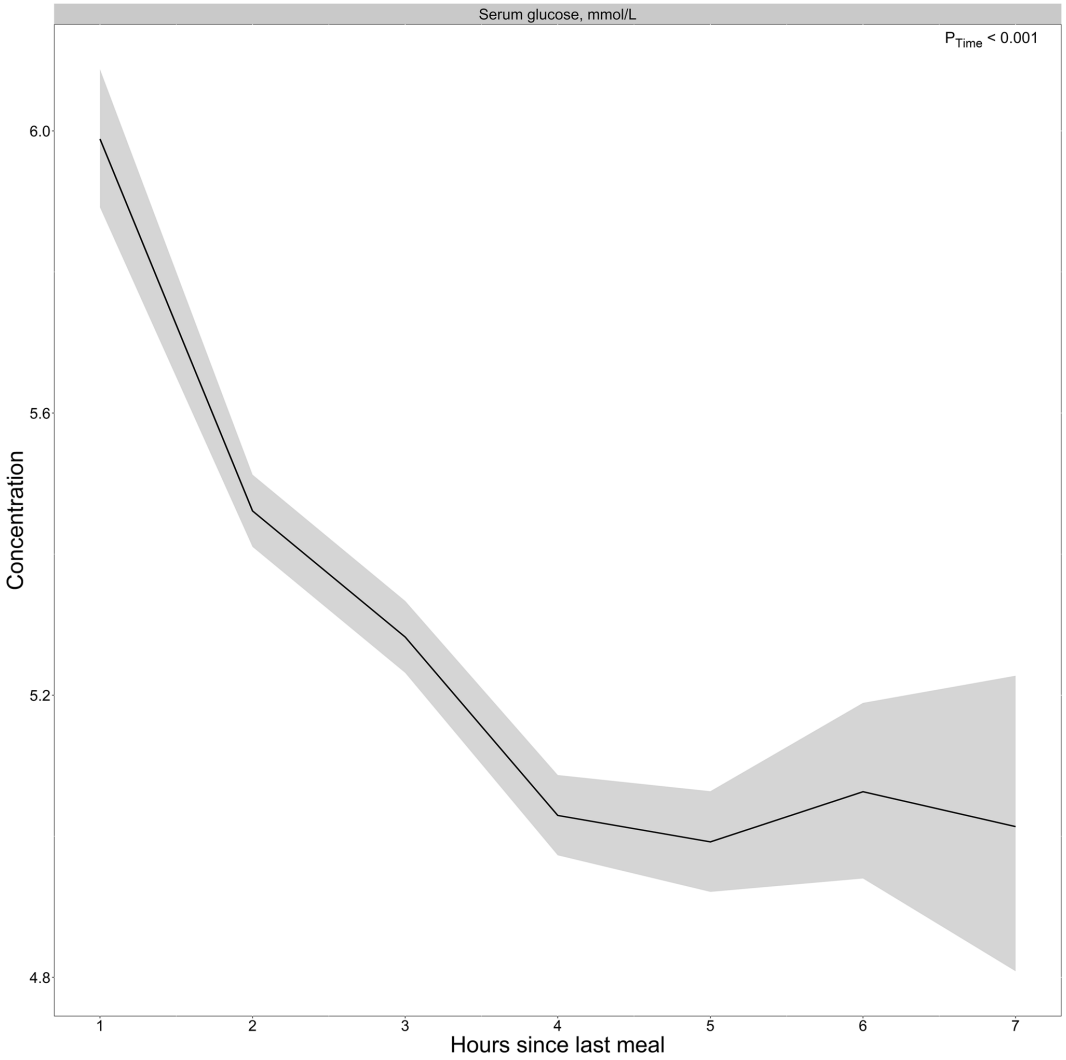

Fig. 2 The concentration of glucose as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis ≠ 0. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4

(Supplementary Fig. 9) and both sexes (Supplementary Fig. 10).

B-vitamin status

Results for the B-vitamin markers are given in Fig. 7 and Table 2. We observed that the concentrations of thiamine and TMP were highest in the first 2 h after a meal (3.42 nmol/L and 7.69 nmol/L, respectively), before steadily declining to their lowest concentrations at 6–7 h (2.60 nmol/L and 6.34 nmol/L), giving a difference between the highest and the lowest values of 31.5% for thiamine and 21.3% for TMP. For FMN, the opposite was true, with the lowest concentration observed during the

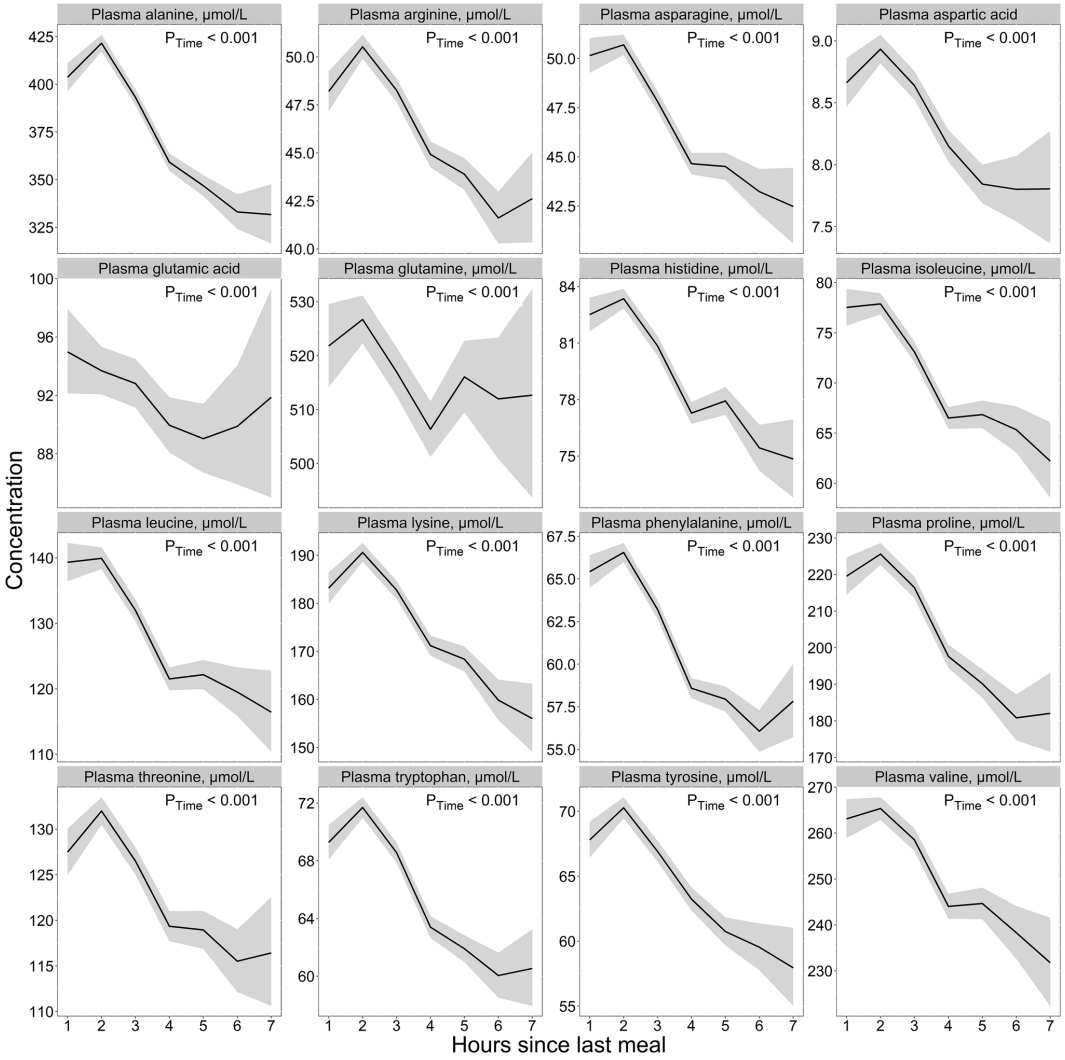

Fig. 3 The concentration of amino acids as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis ≠ 0, and the *y*-axes are scaled to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4

first 2 h after a meal (12.1 nmol/L), and higher concentrations being observed with increasing time since dietary intake, with the highest concentrations observed at 6–7 h after dietary intake (15.2 nmol/L). No clear patterns emerged for the other B-vitamin biomarkers, including riboflavin, nicotinamide, methyl nicotinamide, pyridoxal, PLP, 4-pyridoxic acid, folate, cobalamin, or MMA. Findings were consistent in both age groups (Supplementary Fig. 11) and both sexes (Supplementary Fig. 12).

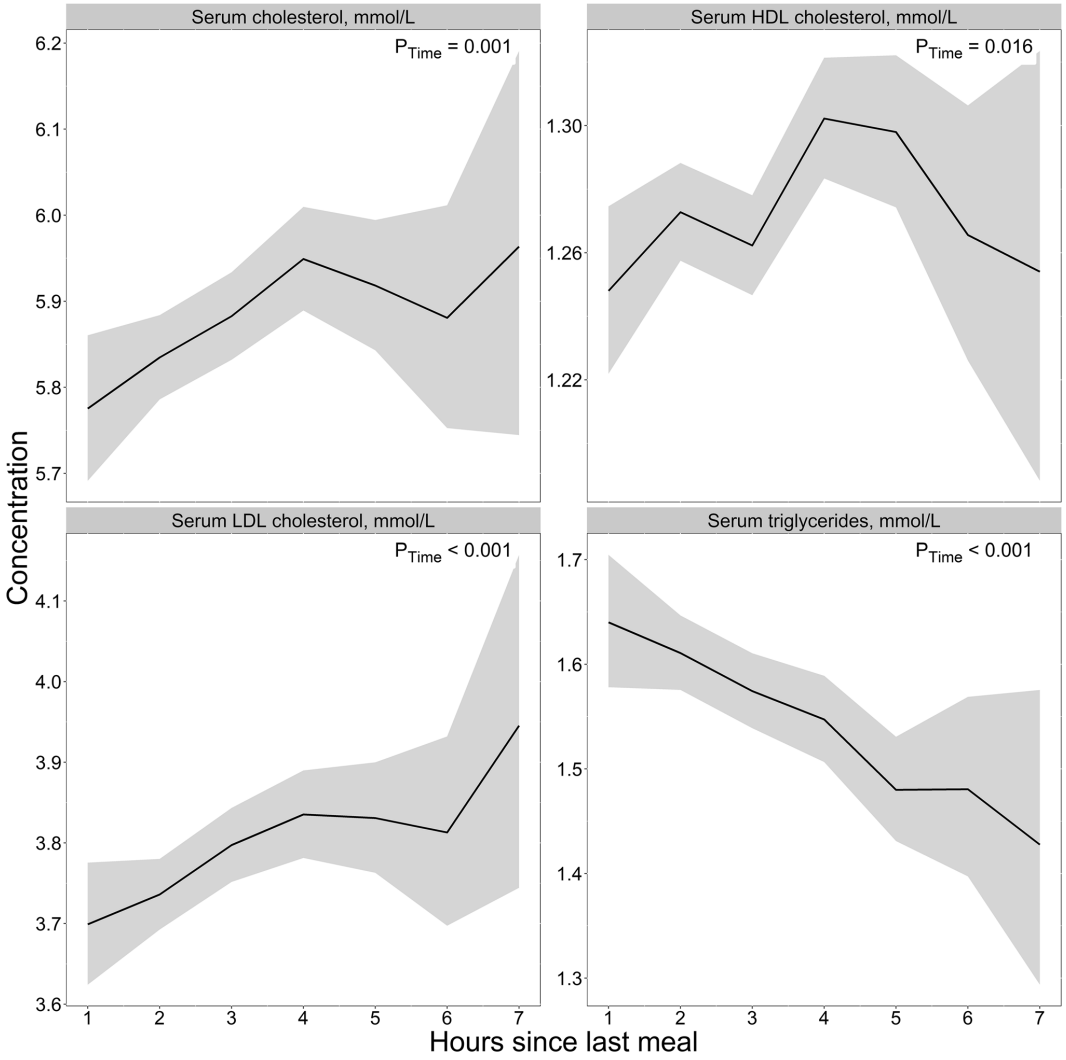

Fig. 4 The concentration of blood lipids as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis ≠ 0, and the *y*-axes are scaled to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4. *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

Discussion

In this study, using cross-sectional data from a large Norwegian cohort including two distinct age groups of community-dwelling adults, we investigated circulating metabolite concentrations as a function of time since last meal. For most amino acids, we observed highest concentrations during the first 3 h after a meal, which was also observed for the one-carbon metabolites betaine, choline, cystathionine, dimethylglycine, glycine, methionine, and serine. Among the lipids, we observed the lowest concentrations of total and LDL cholesterol and the highest concentrations of

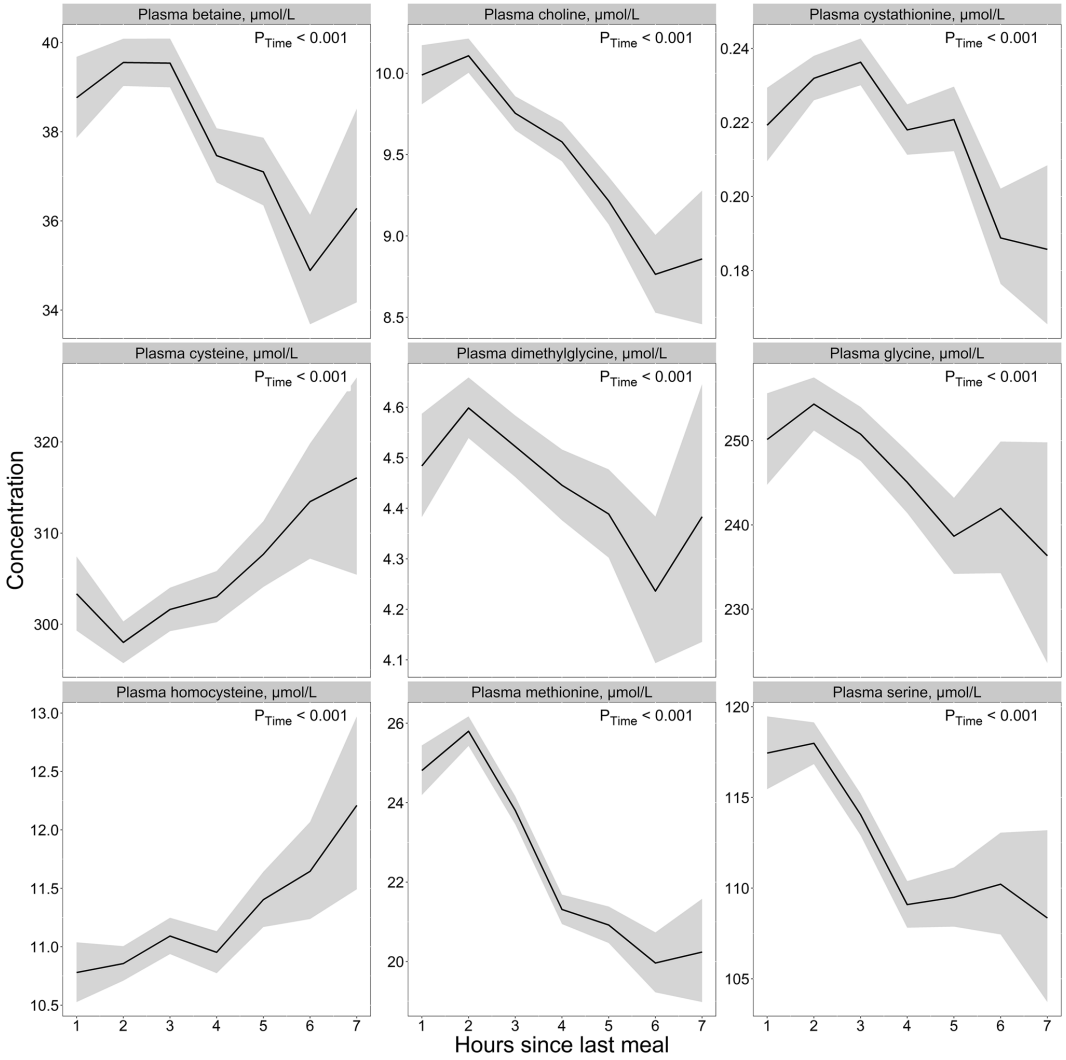

Fig. 5 The concentration of one-carbon metabolites as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis $\neq 0$, and the *y*-axes are scaled to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4

triglycerides the first 2 h after a meal. Lastly, we observed the lowest concentrations of FMN and the highest concentrations of thiamine, TMP, and phylloquinone during the first 2 h after the last meal.

Our findings for glucose are consistent with what is already known from the previous literature, with peak glucose concentrations ~ 1 h after the start of a meal, returning to preprandial levels within a few hours [24]. Thus, the results for glucose may be used as a validation marker for the other results. Further, our amino acid observations are consistent with findings reported elsewhere. However, most of these studies reported concentrations following the

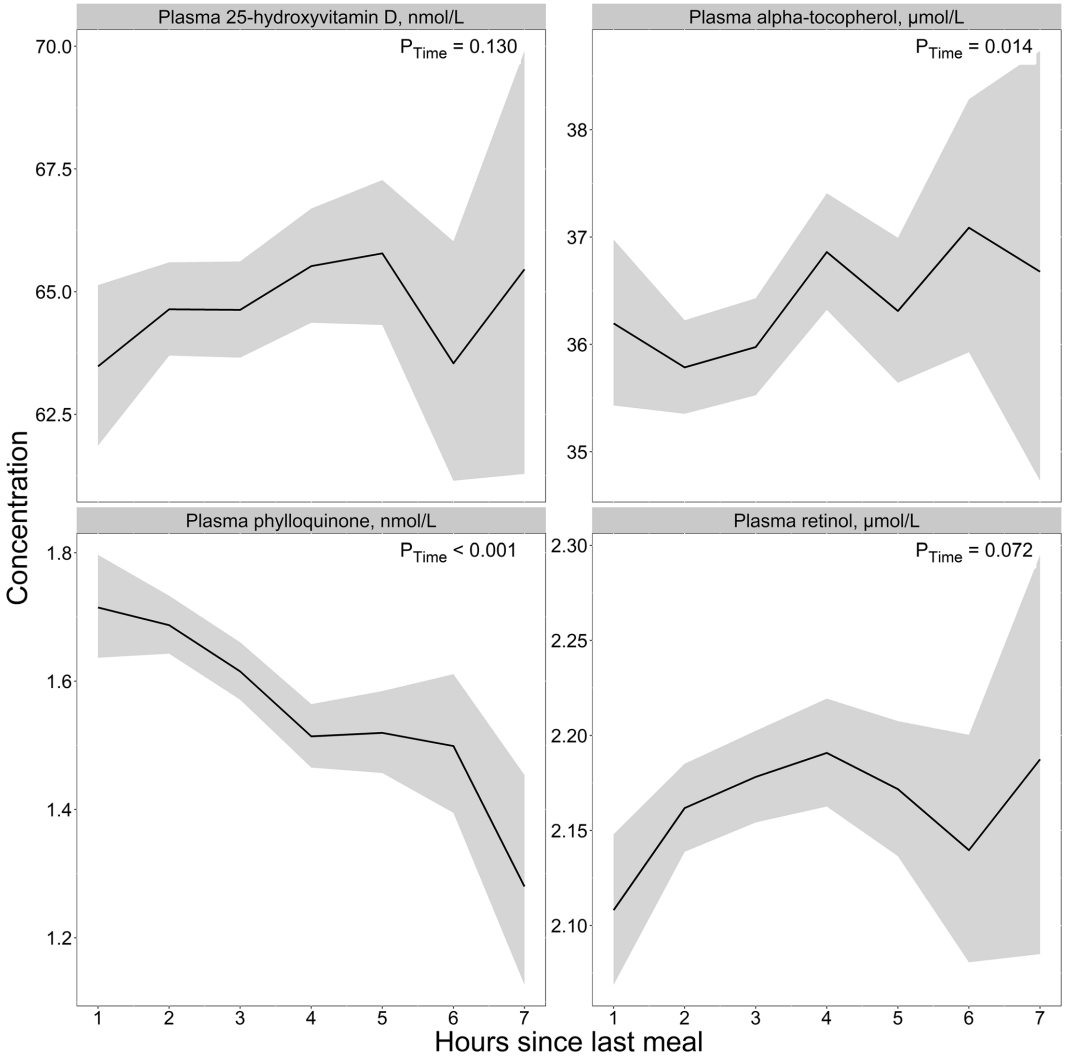

Fig. 6 The concentration of lipid-soluble vitamins as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis ≠ 0, and the *y*-axes are scaled to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4

ingestion of specific foods or nutrients, for instance, dairy products [25], or comparing different types or amounts of protein [26–29]. The results from the present study indicate that circulating amino acid concentrations likely change after a habitual meal. In light of established knowledge about protein metabolism, wherein proteins are cleaved into amino acids which are transported in blood after absorption, these findings are arguably, as expected [30]. Our observations concerning the B-vitamin biomarkers are also in line with previously published reports. In an intervention study, comparing circulating B-vitamin concentrations 5 h after two different meals, Sharma et al. [31] reported that the

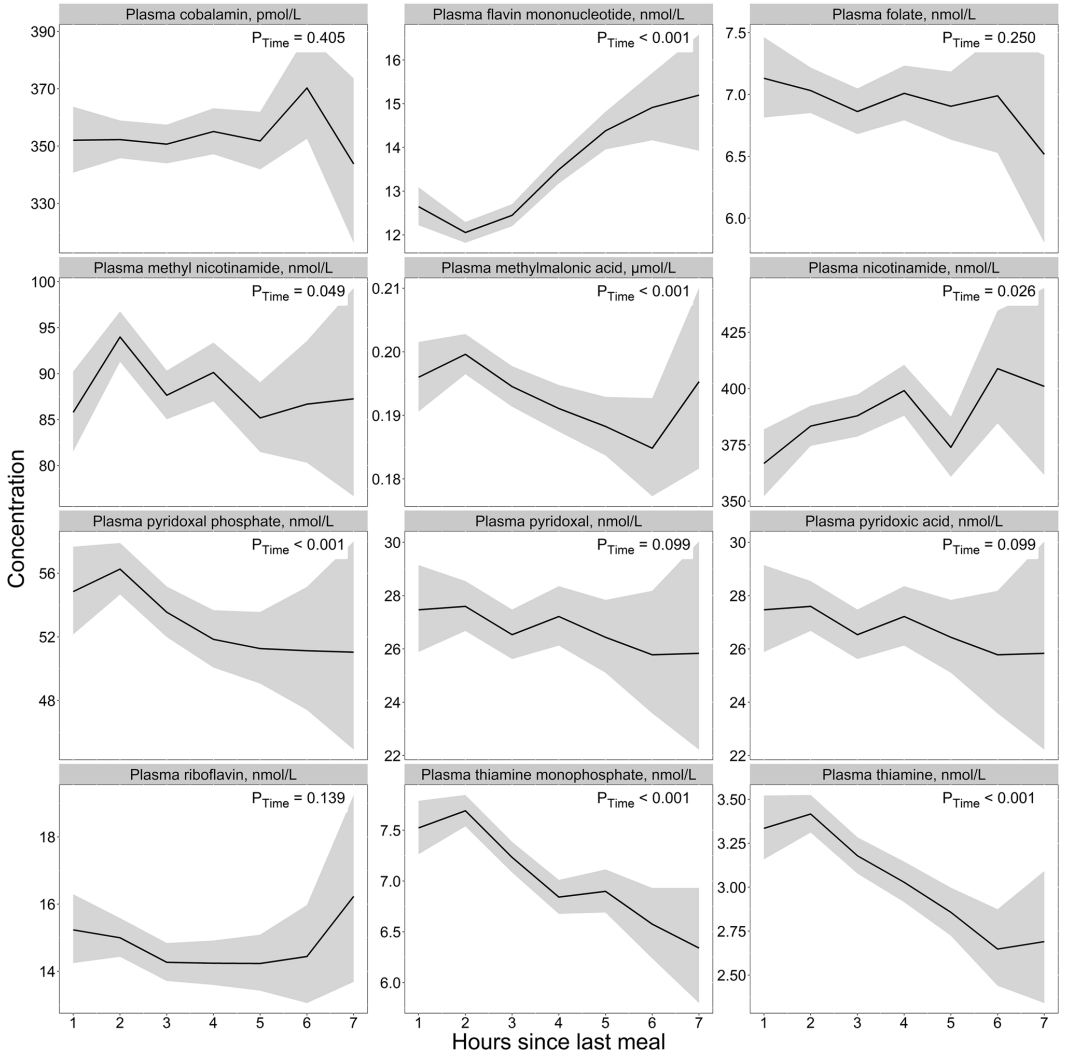

Fig. 7 The concentration of B-vitamin biomarkers as a function of time since last meal using cross-sectional data from 5834 participants in the Hordaland Health Study 1997–1999. The solid line indicates estimated marginal geometric means (from a linear regression model adjusted for age cohort, sex, and BMI), while the shaded area represents 95% geometric confidence intervals. The *p*-value indicated in the figure is for time since last dietary intake. Note, the origin of the *y*-axis ≠ 0, and the *y*-axes are scaled to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint, and the number of missing observations for each metabolite at each timepoint is provided in Supplementary Table 4

concentrations of thiamine were the lowest right after an overnight fast, with the concentrations peaking in the first 2 h after a meal and decreasing thereafter until 5 h after a meal, which was also observed in the present study. The peak in both thiamine and TMP during the first 2 h after a meal, as observed in the present study, is likely attributable to thiamine content in the food, as both free thiamine and TMP enter the bloodstream during absorption of thiamine [32]. For FMN, Sharma et al. reported the highest concentrations immediately before a meal, decreased concentrations immediately after meal ingestion, and increased concentrations thereafter, comparable to the present findings. FMN

serves as a cofactor in the electron transport chain, and the lower concentrations during the 1st hour after a meal may indicate increased utilization of FMN as a cofactor. Comparable to the present study, Sharma et al. also observed relatively stable concentrations of the Vitamin B6 vitamers (pyridoxal, PLP, and pyridoxic acid) [31]. For postprandial lipid concentrations, most studies have investigated the response to high-fat meals [33–36]. However, some studies have investigated blood lipid profiles following non-standardized meals. Using cross-sectional data from 33.391 participants in the Copenhagen General Population Study, Langsted et al. [37] reported the highest concentrations of triglycerides during the 1st hours after a meal, with a maximum mean difference of 0.3 mmol/L, which is comparable to the present findings (0.21 mmol/L). Peak concentrations directly after a meal are likely attributable to fat intake from the meal [37, 38]. Lower concentrations of total and LDL cholesterol the 1st hours after a meal, as observed in the present study, have also been reported by others [37, 39]. Langsted et al. suggested that the observed drop in concentrations could be caused by a hemodilution effect from fluid intake in relation to the meal [37]; however, it has been argued that mechanisms other than hemodilution must be involved [39, 40]. One possible explanation may be attributed to increased hydrolyzation of triglycerides in chylomicrons after a meal, catalyzed by the enzyme lipoprotein lipase (LPL), which subsequently inhibits the formation of LDL from very low-density lipoproteins (VLDLs), as VLDL and chylomicrons compete for LPL [41, 42].

It is crucial to mention that although the findings from the present study indicate that the concentrations of several metabolites change the first 7 h after a meal, these potential changes are not necessarily good, bad, or abnormal. Metabolite concentrations are not static but fluctuate during the day, *e.g.,* after dietary intake, reflecting normal biological variations. With that said, knowing how metabolite concentrations change after a meal is crucial when interpreting metabolite data. In this study, we observed a maximum mean difference of 0.18 mmol/L for total cholesterol, 0.25 mmol/L for LDL cholesterol, and 0.21 mmol/L for triglycerides. These differences are evaluated to be clinically insignificant, as stated by the joint consensus statement from the European Atherosclerosis Society and the European Federation of Clinical Chemistry and Laboratory Medicine in 2016 [10]. However, it is common in clinical practice today to use cutoffs to diagnose or initiate a treatment, and even small changes in concentrations may cause a patient to cross the given cutoff. Thus, if relying on a single measurement, a patient may risk being classified as diseased or non-diseased or given treatment depending on the time since last meal at the time of blood sampling.

Further, the information obtained from this study may be of importance in research settings, where biomarkers may be used to evaluate the effect of a supplement or a treatment, or in epidemiological studies, where metabolites may be modeled as exposures, outcomes, or confounders of associations. In these situations, even modest differences in metabolite concentrations could be of importance. For instance, we observed consistently higher values of plasma homocysteine with increasing time since last dietary intake, with a maximum mean difference of 1.07 µmol/L (13%). Homocysteine has been extensively investigated in epidemiological studies as a risk factor or a marker of a variety of diseases including cardiovascular disease [43] and dementia [44], among others. Similarly, results from epidemiological studies suggest that the branched chained amino acids (BCAAs; leucine, isoleucine, and valine) are biomarkers for increased risk of diabetes [45]. In this study, we observed a maximum difference in leucine of 24 µmol/L (20.7%), isoleucine of 15.7 µmol/L (25.2%), and valine of 33 µmol/L (14.2%). In existing epidemiological studies on the association between the BCAAs and diabetes, information on prandial status at the time of blood sampling is rarely reported [45]. Here, we argue that blood sampling at random hours after a meal may give rise to uncertainty in the results when investigating the association between several metabolites in associations with diseases. Also, when the metabolite concentration changes in the hours after food intake, and the timing of blood collection relative to food intake differs systematically across baseline risk (i.e., blood samples from higher-risk individuals being collected shorter or longer after the last meal), this could attenuate or accentuate the observed association. It is common in epidemiological studies today to distinguish between blood samples taken in the so-called "non-fasting" and "fasting" states, usually based on a cutoff at 6, 8, or 12 h since last meal. In many studies, only metabolites measured in blood samples taken more than 6 or 8 h since last dietary intake are included. However, using cutoffs to categorize an underlying continuous variable assumes homogeneity within the categories, with a sharp discontinuity at the cutoff [46]. Krüg et al. [47] have previously demonstrated that several metabolites change during prolonged fasting. Our findings indicate that many metabolite concentrations change considerably within the first 7 h after dietary intake, which would usually be classified as the "non-fasting" state, among them many metabolites regarded as risk factors for non-communicable diseases. Thus, our findings suggest that it may not be sufficient only to account for dietary intake by distinguishing between the fasting and the non-fasting state but should be done by accounting for the exact time since last meal. As stated, measuring metabolites in the postprandial state could be a better measure of true exposure when using metabolites data in epidemiological studies. When using existing epidemiological data, researchers could, if the information is available, consider adjusting for the exact number of hours since the last meal when modeling

metabolites as an exposure or an outcome. This could reduce the excess variation introduced by collecting blood samples at different time since last meal. When collecting data in the future epidemiological studies, the time of blood sampling should be standardized as much as possible with regard to the time of day and time after dietary intake, and preferably after a standardized meal, to reduce external influence on metabolite concentrations. Should standardization not be possible, then accurate recording of time since last meal for all participants is imperative.

The major strength of this study is the large sample size, including nearly 6000 participants. Further, the narrow age range in the two age cohorts (46–49 and 70–74 years) is considered a strength in this study, as age may affect metabolite concentrations, and thus contributes to variability in the results. The importance of including different age groups is also supported by the observation of, on average, different concentrations according to age for some of the metabolites. The inclusion of both males and females may also be considered a strength of the present study in terms of generalizability. However, usually, there are sex differences in absolute energy and nutrient intakes, which may have driven some of the differences between males and females that we observed for some metabolites. An evident limitation of the study is the cross-sectional design with only a single blood sample from each participant which did not allow for investigation of within-individual changes in metabolite concentrations. Thus, the results must be interpreted as patterns, rather than changes in metabolite concentrations in the postprandial period. Prior to blood sampling, no preparatory instructions were given to the participants on what or when to eat. Thus, the blood samples were taken after meals of varying composition, and no information concerning what the participants ate before the blood sampling was available. The lack of a standardized meal prior to blood sampling may have introduced variability to the results [48]. However, the absence of a standardized meal in the present study could also be interpreted as a strength as it represents a "real-world" setting where blood sampling is usually conducted without any preparatory instructions to the patient or participants on what to eat. The blood sampling was conducted at different times of day and during different seasons of the year. It has been reported that some metabolites exhibit a circadian rhythm, with lipids and amino acids as the most frequently observed rhythmic metabolites [49]. It is also well-known that in populations living far from the Earth´s equator, like Norway, the population concentrations of 25-hydroxyvitamin D, tend to change during seasons [50]. However, there is no reason to believe that there are systematic differences in time of day or season of blood sampling distributed across the time since last meal categories. Thus, we may assume that these are sources of random error and give rise to natural variability in the results. Lastly, the number of observations in the different time categories varied, with a lower number of observations 5–7 h after a meal, making the results at these timepoints subject to greater uncertainty.

Conclusion

In this study of community-dwelling Norwegian middle-aged and elderly adults, we observed patterns among most amino acids and one-carbon metabolites with peak concentrations occurring in the 1st hours after a meal. Concentrations of homocysteine and cysteine were lowest right after the meal, peaking at 6–7 h. The concentrations of phylloquinone and triglycerides were highest 1 h after dietary intake. Thiamine and TMP concentrations were highest, while the concentration of FMN was lowest within the first 2 h after a meal. No clear patterns emerged for the other fat-soluble vitamins, blood lipids, or B-vitamin biomarkers. Our findings indicate that many metabolites and biomarkers change during the first 7 h after a habitual meal, and suggest that the current practice of broadly distinguishing between fasting and non-fasting blood samples in clinical and research settings may be imprecise and inadequate. If confirmed in the future studies, this may have implications for how to account for dietary intake and time since last meal when using existing data, and for the collection of blood samples in the future studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00394-023-03211-y.

Authors contributions The authors' contributions were as follows—ÅMA and VL analyzed data and performed statistical analyses; ÅMA wrote the paper; PMU and AU analyzed the one-carbon metabolites; and all authors (ÅMA, HRR, ON, GST, PMU, AU, AMC, JD, and VL) read and approved the final manuscript.

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Data availability Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

Ethics The Hordaland Health Study was carried out in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics (REC, REC No. 2009/825) and the Norwegian Data Inspectorate. All participants provided written informed consent. The analyses presented here were approved by REK (REK No. 184165).

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Supplementary Material: European Journal of Nutrition

Exploratory analyses on the effect of time since last meal on patterns of amino acids, lipids, one carbon metabolites, and vitamins in the Hordaland Health Study

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Supplementary Table 1. Main characteristics of the analytical platforms used to analyze the amino acids, one-carbon metabolites, and vitamin biomarkers in the Hordaland Health Study 1997-99[1]

Platform	**GC**	**LC1**	**LC2**	**LC3**
Type of analysis	GC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
Metabolites analyzed (year of analysis)	Alanine (2017) Arginine (2017) Asparagine (2017) Aspartic acid (2017) Glutamic acid (2017) Glutamine (2017) Histidine (2017) Isoleucine (2017) Leucine (2017) Lysine (2017) Phenylalanine (2017) Proline (2017) Threonine (2017) Tryptophan (2010) Tyrosine (2017) Valine (2017) Methyl-malonic acid (2017) Cysteine (2010) Cystathionine (2010)	Betaine (2010) Choline (2010) DMG (2010)	Thiamine (2017) Thiamine monophosphate (2017) Riboflavin (2010) Flavin mononucleotide (2010) Nicotinamide (2017) Methyl nicotinamide (2017) Pyridoxal (2010) Pyridoxal-5´-phosphate (2010) 4´-pyridoxic acid (2010)	Retinol (2017) 25-hydroxyvitamin D (2017) α-tocopherol (2017) Phylloquinone (2017)

	Glycine (2017) Homocysteine (2010) Methionine (2010) Serine (2017)			
Pretreatment	Dithioerythriol	-	-	-
Protein precipitation	Ethanol	Trichloroacetic acid	Trichloroacetic acid	Ethanol
Liquid-liquid extraction	Isooctane/chloroform	-	-	Isooctane/chloroform
Derivatization	Methylchloroformate	-	-	-
Column	CP Sil 24 CB	150x4.6 mm, 3 um, phenyl	150x4.6 mm, 3.5 um, C8	50x4.6 mm, 2.7 um, C18
Mobile phase	Helium	Acetic acid:methanol	Acetic acid:HFBA:acetonitrile	Methanol:NH_4-formate
Elution	Temperature step gradient	Isocratic	Step gradient	Isocratic
MS, ion source	Electrospray ionization, positive mode	Electrospray ionization, positive mode	Electrospray ionization, positive mode	Atmospheric chemical ionization, positive mode

[1]All analyses were conducted at Bevital AS (http://bevital.no/). **Abbreviations:** GC-MS, Gas chromatography mass spectometry; HFBA, Heptafluorobutyric acid; LC-MS, liquid chromatography mass spectometry

Supplementary Table 2. The geometric means of metabolite concentrations at each timepoint in middle-aged (M, *n* = 2960) and elderly (E, *n* = 2874) adults in the Hordaland Health Study 1997-1999[1]

Hours after meal		**0-<1**	**1-<2**	**2-<3**	**3-<4**	**4-<5**	**5-<6**	**6-<7**
***n* participants**	M[2]	369	916	731	514	276	113	41
	E[2]	168	712	803	606	425	121	40
Serum glucose, mmol/L	M	5.67	5.19	5.04	4.88	4.87	4.99	5.03
	E	6.38	5.73	5.50	5.17	5.15	5.14	5.00
Amino acids								
Plasma alanine, µmol/L	M	390	406	374	347	334	324	326
	E	412	434	408	367	359	342	338
Plasma arginine, µmol/L	M	47.4	50.3	47.3	43.7	43.0	40.2	43.4
	E	48.1	50.5	48.9	45.6	44.3	42.9	41.8
Plasma asparagine, µmol/L	M	51.1	51.9	48.4	45.3	45.3	44.1	43.0
	E	49.2	49.5	47.2	43.9	43.3	42.2	41.9
Plasma aspartic acid, µmol/L	M	8.84	9.06	8.75	8.27	7.95	8.35	7.70
	E	8.34	8.73	8.39	7.89	7.66	7.28	7.91
Plasma glutamic acid, µmol/L	M	88.8	87.6	87.0	85.3	82.5	84.7	82.9
	E	99.9	98.3	96.0	92.0	94.9	94.9	102
Plasma glutamine, µmol/L	M	517	520	508	496	505	496	498
	E	521	533	524	514	522	526	528
Plasma histidine, µmol/L	M	84.6	86.1	82.5	79.4	79.8	77.2	75.3
	E	80.2	80.3	78.6	74.7	75.6	73.6	74.4
Plasma isoleucine, µmol/L	M	75.7	76.0	70.1	65.3	65.9	65.5	60.6
	E	77.9	78.4	74.1	65.9	67.3	64.9	63.9
Plasma leucine, µmol/L	M	138	138	128	121	122	121	115
	E	138	139	132	119	121	117	118
Plasma lysine, µmol/L	M	179	188	176	168	164	157	150
	E	186	191	188	173	172	162	162
Plasma phenylalanine, µmol/L	M	63.3	64.6	61.3	57.4	56.6	55.2	56.6
	E	67.8	68.2	64.7	59.4	59.3	57.0	59.1
Plasma proline, µmol/L	M	212	217	210	191	181	179	169
	E	222	232	218	198	196	181	195
Plasma threonine, µmol/L	M	132	137	128	123	124	120	124
	E	122	127	124	115	114	111	109
Plasma tryptophan, µmol/L	M	70.4	73.3	69.1	64.6	62.1	61.9	63.5
	E	67.4	69.4	67.0	61.3	60.8	58.1	57.6
Plasma tyrosine, µmol/L	M	64.0	67.0	63.6	60.5	58.2	56.6	55.6

	E	72.5	73.4	70.1	66.0	63.7	62.7	60.4
Plasma valine, µmol/L	M	257	259	250	241	241	238	227
	E	266	268	262	242	248	238	237
Blood lipis								
Serum total cholesterol, mmol/L	M	5.57	5.61	5.63	5.68	5.66	5.68	5.79
	E	5.98	6.09	6.19	6.24	6.23	6.11	6.14
Serum LDL-cholesterol, mmol/L	M	3.53	3.54	3.57	3.66	3.62	3.65	3.83
	E	3.86	3.95	4.05	4.04	4.08	3.99	4.06
Serum HDL-cholesterol, mmol/L	M	1.25	1.29	1.27	1.28	1.28	1.22	1.25
	E	1.28	1.28	1.30	1.37	1.33	1.31	1.26
Serum triglycerides, mmol/L	M	1.54	1.49	1.46	1.48	1.40	1.44	1.29
	E	1.71	1.71	1.64	1.58	1.59	1.52	1.58
One-carbon metabolites								
Plasma betaine, µmol/L	M	36.4	37.5	37.4	34.4	35.5	34.2	35.9
	E	40.4	40.9	40.3	38.7	37.6	35.1	36.5
Plasma choline, µmol/L	M	9.46	9.58	9.20	9.01	8.66	8.48	8.24
	E	10.4	10.6	10.2	9.99	9.70	9.03	9.52
Plasma cysteine, µmol/L	M	288	281	285	285	295	294	293
	E	316	316	318	321	323	335	341
Plasma cystathionine, µmol/L	M	0.18	0.19	0.20	0.18	0.18	0.16	0.15
	E	0.26	0.28	0.27	0.26	0.27	0.23	0.22
Plasma dimethylglycine, µmol/L	M	4.39	4.52	4.47	4.33	4.34	4.31	4.37
	E	4.55	4.63	4.50	4.46	4.38	4.15	4.39
Plasma glycine, µmol/L	M	255	258	254	249	241	237	244
	E	248	254	254	248	238	248	228
Plasma homocysteine, µmol/L	M	9.73	9.72	9.92	9.77	10.3	10.5	10.8
	E	11.7	12.0	12.1	12.0	12.5	12.9	13.7
Plasma methionine, µmol/L	M	24.7	25.9	23.4	21.1	21.0	20.1	20.5
	E	24.7	25.4	23.8	21.1	20.6	19.8	20.0
Plasma serine, µmol/L	M	120	121	116	112	113	113	115
	E	115	116	113	108	106	108	102
Lipid-solube vitamins								
Plasma retinol, µmol/L	M	2.08	2.13	2.15	2.15	2.11	2.11	2.12
	E	2.10	2.17	2.16	2.19	2.20	2.16	2.26
Plasma 25-OH-vitD, nmol/L	M	61.3	62.5	62.6	64.4	64.7	62.2	60.6
	E	66.3	66.9	66.8	66.8	66.6	64.7	70.7
Plasma α-tocopherol, µmol/L	M	34.4	34.0	34.2	35.1	34.7	34.8	34.9
	E	38.4	37.8	38.3	39.2	38.5	39.7	38.6
Plasma phylloquinone, nmol/L	M	1.64	1.62	1.58	1.46	1.48	1.53	1.32

	E	1.78	1.74	1.62	1.54	1.55	1.47	1.24
B-vitamin biomarkers								
Plasma thiamine, nmol/L	M	3.12	3.21	3.00	2.94	2.65	2.30	2.39
	E	3.69	3.72	3.52	3.30	3.17	3.08	3.04
Plasma TMP, nmol/L	M	7.74	8.01	7.40	7.04	6.96	6.66	6.47
	E	7.44	7.49	7.29	6.91	6.90	6.56	6.22
Plasma riboflavin, nmol/L	M	14.2	13.5	12.9	13.2	12.6	12.4	14.2
	E	16.3	16.9	16.3	15.9	16.4	17.0	18.6
Plasma FMN, nmol/L	M	13.0	12.5	13.1	14.2	14.7	15.4	16.3
	E	12.5	11.7	11.9	12.9	14.0	14.5	14.2
Plasma nicotinamide, nmol/L	M	395	390	403	421	394	402	382
	E	328	380	374	382	361	415	421
Plasma methyl nicotinamide, nmol/L	M	82.4	87.4	83.2	86.0	79.9	75.8	64.0
	E	88.8	103	94.9	97.8	93.2	99.7	103
Plasma pyridoxal, nmol/L	M	13.1	13.3	12.5	12.1	11.9	11.8	11.0
	E	15.5	15.2	14.2	13.7	13.5	13.7	15.8
Plasma PLP, nmol/L	M	55.1	57.5	53.7	52.9	51.1	51.5	46.7
	E	55.1	55.0	53.7	51.2	51.3	50.8	55.9
Plasma 4-pyridoxic acid, nmol/L	M	23.1	23.5	22.9	23.7	22.9	21.1	19.2
	E	33.5	32.6	31.3	31.8	31.0	31.4	35.0
Plasma folate, nmol/L	M	7.23	7.02	6.84	7.01	6.78	6.64	6.62
	E	7.01	7.12	7.05	7.21	7.09	7.39	6.42
Plasma cobalamin, pmol/L	M	353	355	355	363	355	381	367
	E	358	350	348	349	350	361	322
Plasma MMA, µmol/L	M	0.18	0.18	0.18	0.17	0.17	0.16	0.17
	E	0.22	0.22	0.22	0.22	0.21	0.21	0.23

[1]All values are presented as geometric means. An overview of missing observations at each timepoint for each of the metabolites can be found in Supplementary Table 4. [2]Middle-aged group (aged 46-49 years) / Elderly group (aged 70-74 years). **Abbreviations**: FMN, flavin mononucleotide; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; MMA, methyl-malonic acid; PLP, pyridoxal 5'-phosphate; TMP, thiamine monophosphate

Supplementary Table 3. The geometric means of metabolite concentrations the first seven hours after a meal in males (*n* = 2541) and females (*n* = 3293) in the Hordaland Health Study 1997-1999[1]

Hours after meal		**0-<1**	**1-<2**	**2-<3**	**3-<4**	**4-<5**	**5-<6**	**6-<7**
***n* participants**	M[2]	235	743	647	452	313	111	40
	F[2]	302	885	886	668	388	123	41
Serum glucose, mmol/L	M	5.98	5.65	5.39	5.17	5.05	5.01	4.94
	F	5.81	5.23	5.19	4.94	5.02	5.12	5.08
Amino acids								
Plasma alanine, µmol/L	M	410	435	409	377	361	347	328
	F	387	405	379	346	339	321	335
Plasma arginine, µmol/L	M	50.1	51.8	49.3	46.3	44.5	43.7	43.6
	F	46.1	49.2	47.3	43.7	43.3	39.7	41.6
Plasma asparagine, µmol/L	M	50.8	50.9	47.8	44.5	44.4	43.1	42.1
	F	50.2	50.7	47.7	44.5	43.9	43.1	42.8
Plasma aspartic acid, µmol/L	M	9.14	9.41	9.00	8.52	8.32	8.43	7.95
	F	8.35	8.52	8.25	7.78	7.37	7.24	7.67
Plasma glutamic acid, µmol/L	M	105	102	101	99.6	98.6	102	96.8
	F	83.6	84.7	85.2	82.5	83.4	80.5	87.2
Plasma glutamine, µmol/L	M	524	534	523	510	520	515	524
	F	513	518	512	503	512	508	497
Plasma histidine, µmol/L	M	84.8	85.1	82.4	78.6	79.0	77.7	75.6
	F	82.0	82.2	79.1	75.7	75.9	73.2	74.2
Plasma isoleucine, µmol/L	M	83.5	85.5	79.1	71.5	71.8	72.6	65.6
	F	71.4	70.8	67.5	62.0	62.9	59.3	59.0
Plasma leucine, µmol/L	M	151	154	143	131	131	132	122
	F	128	128	122	113	114	109	111
Plasma lysine, µmol/L	M	187	197	188	176	174	165	157
	F	177	184	178	167	165	156	155
Plasma phenylalanine, µmol/L	M	66.4	68.3	65.0	59.6	59.7	57.5	58.2
	F	63.3	64.4	61.6	57.8	57.1	54.9	57.5
Plasma proline, µmol/L	M	232	241	234	215	207	207	197
	F	203	209	200	183	177	160	169
Plasma threonine, µmol/L	M	129	134	128	121	119	119	121
	F	129	131	125	117	117	112	113
Plasma tryptophan, µmol/L	M	73.9	74.6	71.4	66.5	64.5	64.8	61.8
	F	66.3	69.1	65.6	60.4	58.9	55.8	59.3
Plasma tyrosine, µmol/L	M	68.5	71.4	67.7	64.0	61.9	60.4	58.1

	F	65.0	68.4	66.3	63.1	61.1	59.0	57.8
Plasma valine, µmol/L	M	279	285	275	260	261	257	240
	F	246	247	243	230	232	222	224
Blood lipis								
Serum total cholesterol, mmol/L	M	5.60	5.73	5.76	5.79	5.82	5.82	5.89
	F	5.78	5.88	6.03	6.10	6.15	5.97	6.04
Serum LDL-cholesterol, mmol/L	M	3.60	3.73	3.76	3.76	3.85	3.87	3.89
	F	3.65	3.70	3.85	3.93	3.93	3.79	4.00
Serum HDL-cholesterol, mmol/L	M	1.13	1.14	1.14	1.18	1.16	1.11	1.22
	F	1.37	1.42	1.41	1.43	1.43	1.44	1.29
Serum triglycerides, mmol/L	M	1.76	1.79	1.75	1.69	1.60	1.66	1.49
	F	1.47	1.42	1.42	1.43	1.44	1.34	1.37
One-carbon metabolites								
Plasma betaine, µmol/L	M	44.1	44.8	44.4	42.2	42.0	40.3	43.6
	F	33.2	34.6	35.3	33.3	33.1	30.3	30.2
Plasma choline, µmol/L	M	10.2	10.6	10.3	10.2	9.92	9.32	9.39
	F	9.40	0.52	9.31	0.13	8.80	8.29	8.35
Plasma cysteine, µmol/L	M	302	302	307	308	315	310	318
	F	292	289	298	300	309	317	313
Plasma cystathionine, µmol/L	M	0.23	0.25	0.26	0.24	0.25	0.21	0.19
	F	0.19	0.20	0.22	0.20	0.21	0.17	0.18
Plasma dimethylglycine, µmol/L	M	4.71	4.88	4.77	4.65	4.64	4.65	4.61
	F	4.24	4.33	4.28	4.24	4.16	3.88	4.17
Plasma glycine, µmol/L	M	233	234	232	226	222	219	219
	F	269	276	271	265	254	266	254
Plasma homocysteine, µmol/L	M	11.3	11.6	11.9	11.8	13.2	12.8	12.7
	F	9.57	9.91	10.4	10.4	11.0	10.7	11.7
Plasma methionine, µmol/L	M	26.5	27.6	25.3	22.5	21.8	21.0	20.5
	F	23.3	24.2	22.4	20.2	20.0	18.9	20.0
Plasma serine, µmol/L	M	114	114	111	105	105	108	107
	F	122	122	118	112	112	112	109
Lipid-solube vitamins								
Plasma retinol, µmol/L	M	2.21	2.30	2.30	2.32	2.30	2.25	2.25
	F	2.00	2.03	2.06	2.07	2.07	2.04	2.13
Plasma 25-OH-vitD, nmol/L	M	60.8	64.1	64.4	65.4	68.1	62.3	64.3
	F	64.4	64.7	65.1	65.8	64.1	64.6	66.5
Plasma α-tocopherol, µmol/L	M	34.5	34.3	34.8	35.4	35.3	35.7	35.4
	F	36.6	36.8	37.4	38.6	38.3	38.7	38.0
Plasma phylloquinone, nmol/L	M	1.75	1.82	1.72	1.61	1.62	1.61	1.31

	F	1.64	1.56	1.52	1.43	1.45	1.41	1.26
B-vitamin biomarkers								
Plasma thiamine, nmol/L	M	2.76	2.97	2.86	2.69	2.53	2.30	2.29
	F	3.77	3.85	3.60	3.46	3.34	3.06	3.15
Plasma TMP, nmol/L	M	6.80	7.01	6.55	6.21	6.24	6.06	5.85
	F	8.36	8.47	7.97	7.50	7.53	7.12	6.87
Plasma riboflavin, nmol/L	M	13.0	13.8	14.0	12.5	13.0	11.9	13.9
	F	16.3	15.8	15.0	16.2	16.4	17.5	18.8
Plasma FMN, nmol/L	M	12.8	12.0	12.3	12.7	13.5	14.0	14.4
	F	12.9	12.2	12.6	14.0	15.0	15.8	16.1
Plasma nicotinamide, nmol/L	M	366	377	383	392	367	423	395
	F	377	392	391	404	379	396	408
Plasma methyl nicotinamide, nmol/L	M	77.6	85.2	81.8	82.5	80.9	80.3	79.1
	F	90.0	102	94.8	99.2	93.5	94.4	96.1
Plasma pyridoxal, nmol/L	M	13.5	13.8	13.1	12.4	12.2	12.0	12.2
	F	14.1	14.3	13.5	13.3	13.3	13.4	14.2
Plasma PLP, nmol/L	M	54.0	54.8	52.7	51.6	50.3	50.1	49.3
	F	56.0	57.7	54.4	52.2	51.9	52.1	52.8
Plasma 4-pyridoxic acid, nmol/L	M	24.8	25.6	26.2	26.9	26.3	25.4	23.4
	F	27.0	28.5	27.5	28.4	28.5	26.5	28.4
Plasma folate, nmol/L	M	6.34	6.61	6.52	6.40	6.40	5.88	6.43
	F	7.87	7.48	7.29	7.64	7.46	8.24	6.61
Plasma cobalamin, pmol/L	M	354	343	347	351	343	349	345
	F	354	362	355	359	359	391	343
Plasma MMA, µmol/L	M	0.18	0.20	0.20	0.20	0.19	0.20	0.20
	F	0.19	0.20	0.20	0.19	0.19	0.17	0.19

[1]All values are presented as geometric means. An overview of missing observations at each timepoint for each of the metabolites can be found in Supplementary Table 4. [2]Males/females **Abbreviations**: FMN, flavin mononucleotide; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; MMA, methyl-malonic acid; PLP, pyridoxal 5'-phosphate; TMP, thiamine monophosphate

Supplementary Table 4. The number of missing values for each metabolite at each timepoint in the Hordaland Health Study 1997-1999

Hours after meal, h	**1**	**2**	**3**	**4**	**5**	**6**	**7**
n	537	1628	1533	1120	701	234	81
Serum glucose, mmol/L	0	0	0	0	0	0	0
Amino acids							
Plasma alanine, µmol/L	13	23	27	20	13	1	2
Plasma arginine, µmol/L	1	1	2	2	0	0	0
Plasma asparagine, µmol/L	13	27	29	27	18	2	2
Plasma aspartic acid, µmol/L	13	23	27	19	23	1	2
Plasma glutamic acid, µmol/L	13	23	27	19	23	1	2
Plasma glutamine, µmol/L	13	23	28	25	15	2	2
Plasma histidine, µmol/L	13	23	27	20	13	1	2
Plasma isoleucine, µmol/L	13	23	27	20	13	1	2
Plasma leucine, µmol/L	13	23	27	20	13	1	2
Plasma lysine, µmol/L	13	23	27	20	13	1	2
Plasma phenylalanine, µmol/L	14	23	28	25	15	2	2
Plasma proline, µmol/L	14	28	30	24	15	1	2
Plasma threonine, µmol/L	14	24	28	25	15	2	2
Plasma tryptophan, µmol/L	6	9	16	11	4	2	0
Plasma tyrosine, µmol/L	13	23	28	25	15	2	2
Plasma valine, µmol/L	13	23	27	20	13	1	2
Blood lipids							
Serum total cholesterol, mmol/L	0	0	0	0	0	0	0
Serum LDL-cholesterol, mmol/L	0	0	0	0	0	0	0
Serum HDL-cholesterol, mmol/L	0	0	0	0	0	0	0
Serum triglycerides, mmol/L	0	0	0	0	0	0	0
One-carbon metabolites							
Plasma betaine, µmol/L	1	1	2	2	0	0	0
Plasma choline, µmol/L	1	1	2	2	0	0	0
Plasma cysteine, µmol/L	5	17	17	13	8	3	1
Plasma cystathionine, µmol/L	1	1	2	2	0	1	0
Plasma dimethylglycine, µmol/L	1	1	2	2	0	0	0
Plasma glycine, µmol/L	13	23	27	20	13	1	2
Plasma homocysteine, µmol/L	1	1	2	2	0	0	0
Plasma methionine, µmol/L	1	1	2	2	0	0	0
Plasma serine, µmol/L	13	23	27	20	13	1	2
Lipid-soluble vitamins							
Plasma retinol, µmol/L	13	23	30	20	13	1	2
Plasma 25-OH-vitD, nmol/L	13	31	31	21	14	2	2
Plasma α-tocopherol, µmol/L	13	23	30	20	13	1	2
Plasma phylloquinone, nmol/L	48	144	147	121	95	31	16
B-vitamin biomarkers							
Plasma thiamine, nmol/L	13	45	59	49	25	7	2
Plasma TMP, nmol/L	13	45	60	49	25	7	2
Plasma riboflavin, nmol/L	6	9	16	11	6	3	1

Plasma FMN, nmol/L	6	9	16	11	6	3	1
Plasma nicotinamide, nmol/L	13	46	59	50	26	8	2
Plasma methyl nicotinamide, nmol/L	13	45	60	49	25	8	2
Plasma pyridoxal, nmol/L	6	9	16	11	4	2	0
Plasma PLP, nmol/L	6	9	16	11	4	2	0
Plasma 4-pyridoxic acid, nmol/L	6	9	16	11	4	2	0
Plasma folate, nmol/L	1	8	4	6	4	0	0
Plasma cobalamin, pmol/L	0	3	2	3	0	0	0
Plasma MMA, µmol/L	28	54	56	40	40	9	7

Abbreviations: HDL, High density lipoprotein; LDL, Low-density lipoprotein

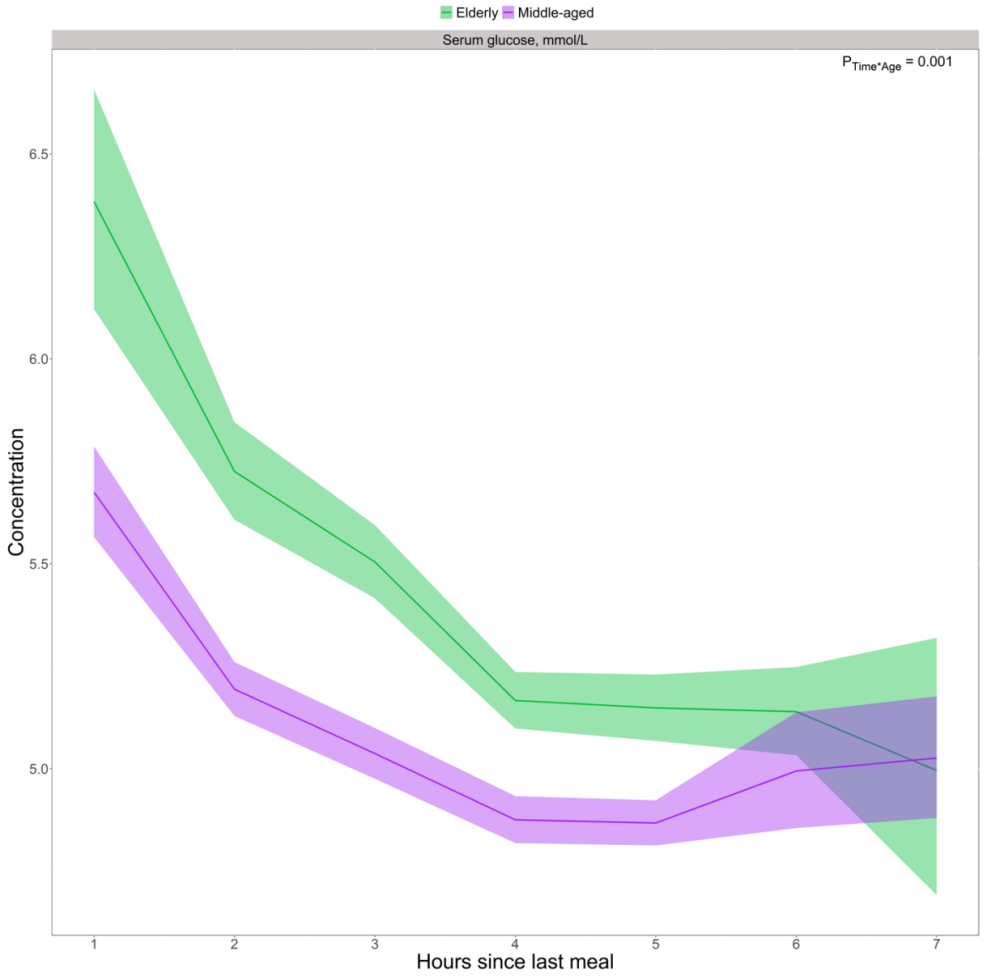
Supplementary Table 5. The number of missing observations at each timepoint in middle-aged and elderly adults, and among males and females in the Hordaland Health Study 1997-1999

	Age cohorts														**Sex**													
	Middle-aged group, aged 46-49 years, *n* = 2960							**Elderly group,** aged 70-74 years, *n* = 2874							**Males,** *n* = 2541							**Females,** *n* = 3293						
Hours after meal	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
***n* participants**	369	916	731	514	276	113	41	168	712	803	606	425	121	40	235	743	647	452	313	111	40	302	885	886	668	388	123	41
Glucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amino acids																												
Alanine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Arginine	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0
Asparagine	10	17	13	15	7	2	1	3	10	16	12	11	0	1	8	18	16	19	10	1	1	5	9	13	8	8	1	1
Aspartic acid	10	16	12	9	15	1	1	3	7	15	10	8	0	1	8	17	15	15	9	1	1	5	6	12	4	4	0	1
Glutamic acid	10	16	12	9	15	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Glutamine	10	16	13	15	7	2	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Histidine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Isoleucine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Leucine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Lysine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Phenylalanine	11	16	13	15	7	2	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	6	6	12	4	4	0	1
Proline	11	20	13	11	6	1	1	3	8	17	13	9	0	1	8	17	15	20	10	1	1	6	8	15	7	5	0	1
Threonine	10	16	13	15	7	2	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Tryptophan	6	8	9	6	1	1	0	0	1	7	5	3	1	0	4	7	8	9	4	1	0	2	2	8	2	0	1	0
Tyrosine	10	16	13	15	7	2	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Valine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1
Lipids																												
Cholesterol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

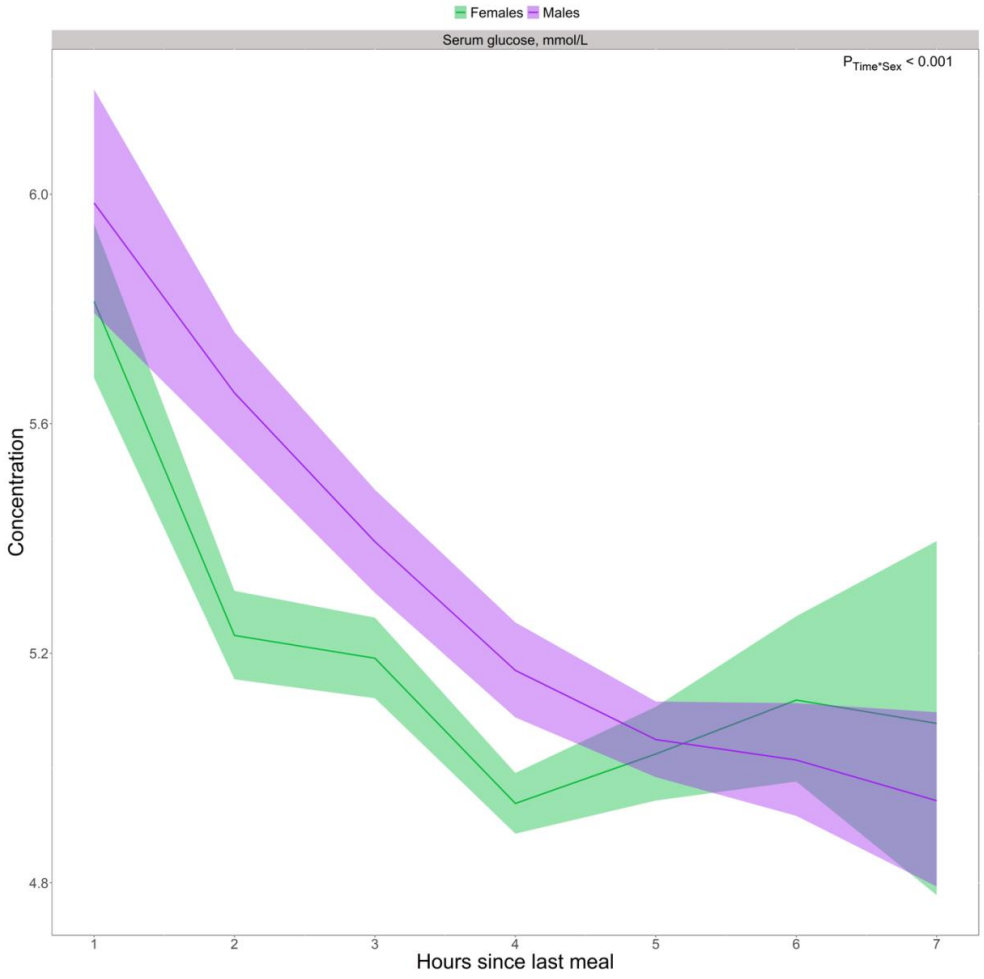
LDL-C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HDL-C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triglycerides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
One-carbon metabolites																													
Betaine	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0	0
Choline	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0	0
Cysteine	1	1	0	1	0	0	0	4	16	17	12	8	3	1	3	8	8	6	3	1	1	2	9	9	7	5	2	0	
Cystathionine	1	1	1	1	0	0	0	0	0	2	1	0	1	0	3	8	8	6	3	1	1	0	0	1	0	0	1	0	
DMG	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0	
Glycine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1	
Homocysteine	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0	
Methionine	1	1	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	2	0	0	0	0	0	0	0	0	0	0	
Serine	10	16	12	10	5	1	1	3	7	15	10	8	0	1	8	17	15	16	9	1	1	5	6	12	4	4	0	1	
Lipid-soluble vitamins																													
Retinol	9	17	14	10	5	1	1	4	7	16	10	8	0	1	4	6	14	4	4	0	1	9	18	16	16	9	1	1	
25-OH-vitD	9	23	15	11	5	2	1	4	8	16	10	9	0	1	4	10	14	4	5	0	1	9	21	17	17	9	2	1	
α-tocopherol	9	17	14	10	5	1	1	4	7	16	10	8	0	1	4	6	14	4	4	0	1	9	18	16	16	9	1	1	
Phylloquinone	40	94	81	69	43	19	8	8	50	66	52	52	12	8	25	88	99	74	58	16	12	23	56	48	47	37	15	4	
B-vitamin biomarkers																													
Thiamine	12	24	28	24	10	5	1	1	21	31	25	15	2	1	6	27	30	28	13	5	1	7	18	29	21	12	2	1	
TMP	12	24	29	24	10	5	1	1	21	31	25	15	2	1	6	27	30	28	13	5	1	7	18	30	21	12	2	1	
Riboflavin	6	8	9	6	1	1	0	0	1	7	5	5	2	1	4	7	8	9	4	1	0	2	2	8	2	0	1	0	
FMN	6	8	9	6	1	1	0	0	1	7	5	5	2	1	4	7	8	9	4	1	0	2	2	8	2	0	1	0	
Nicotinamide	12	24	28	25	10	6	1	1	22	31	25	16	2	1	6	28	30	28	14	5	1	7	18	29	22	12	3	1	
Methyl nicotinamide	12	24	29	24	10	6	1	1	21	31	25	15	2	1	6	27	30	28	13	5	1	7	18	29	21	12	2	1	
Pyridoxal	6	8	9	6	1	1	0	0	1	7	5	3	1	0	4	7	8	9	4	1	0	2	2	8	2	0	1	0	
PLP	6	8	9	6	1	1	0	0	1	7	5	3	1	0	4	7	8	9	4	1	0	2	2	8	2	0	1	0	
4-pyridoxic acid	6	8	9	6	1	1	0	0	1	7	5	3	1	0	4	7	8	9	4	1	0	2	2	8	2	0	1	0	

Folate	1	4	2	1	0	0	0	0	4	2	5	4	0	0	0	6	0	5	3	0	0	1	2	4	1	1	0	0
Cobalamin	0	2	1	1	0	0	0	0	1	1	2	0	0	0	0	3	0	3	0	0	0	0	0	2	0	0	0	0
MMA	22	38	34	20	21	4	5	6	16	22	20	19	5	2	17	26	28	27	21	7	3	11	28	28	13	19	2	4

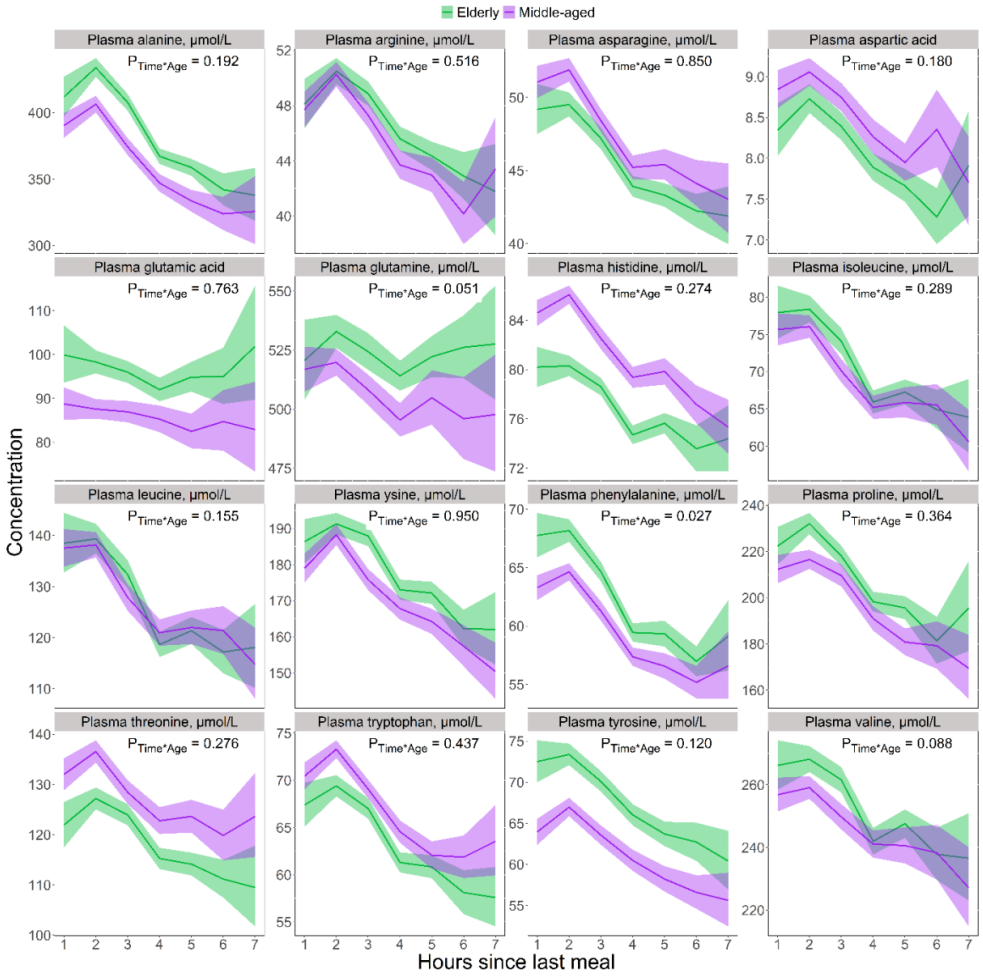
Abbreviations: 25-OH-vitD, 25-hydroxyvitamin D; DMG, dimethylglycine; FMN, Flavin mononucleotide; HDL-C, High density lipoprotein cholesterol; HUSK, Hordaland Health Study; LDL-C, Low-density lipoprotein cholesterol; MMA, methylmalonic acid; PLP, Pyridoxal-5'-phosphate, TMP, Thiamine monophosphate



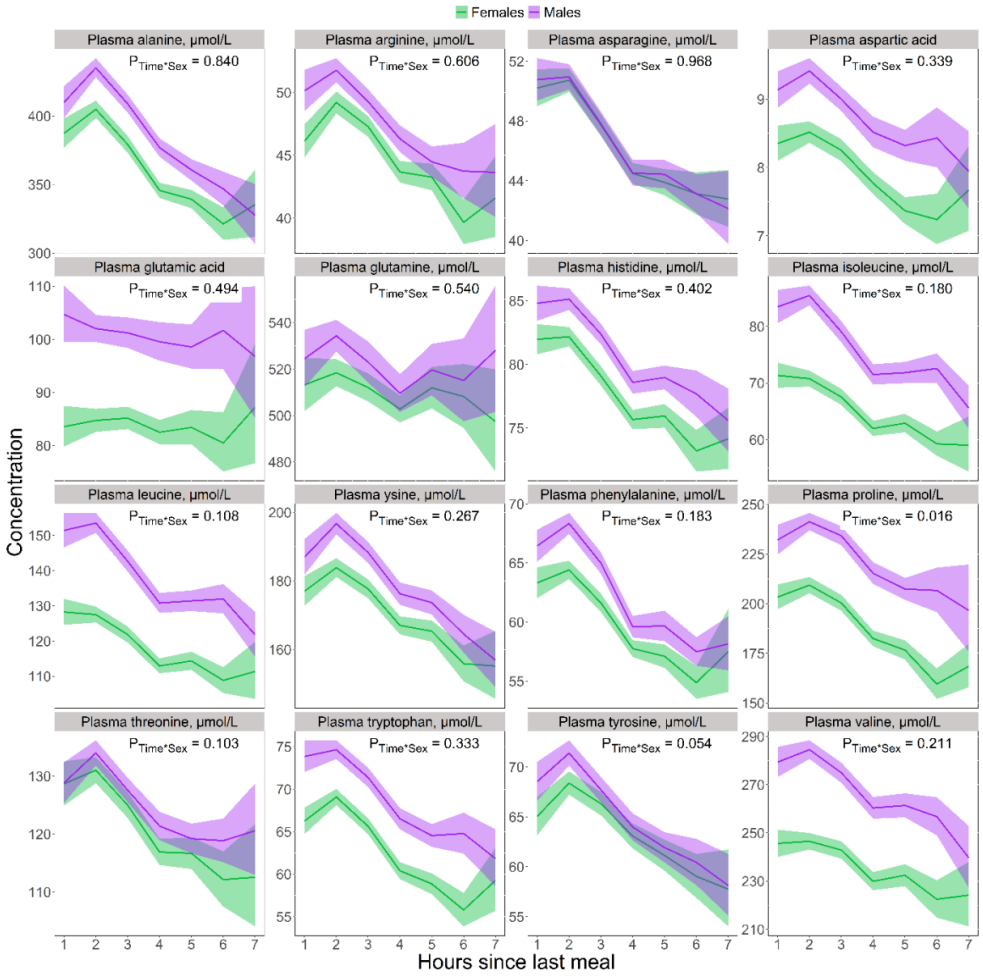
Supplementary Figure 1. The concentration of glucose as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represents the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



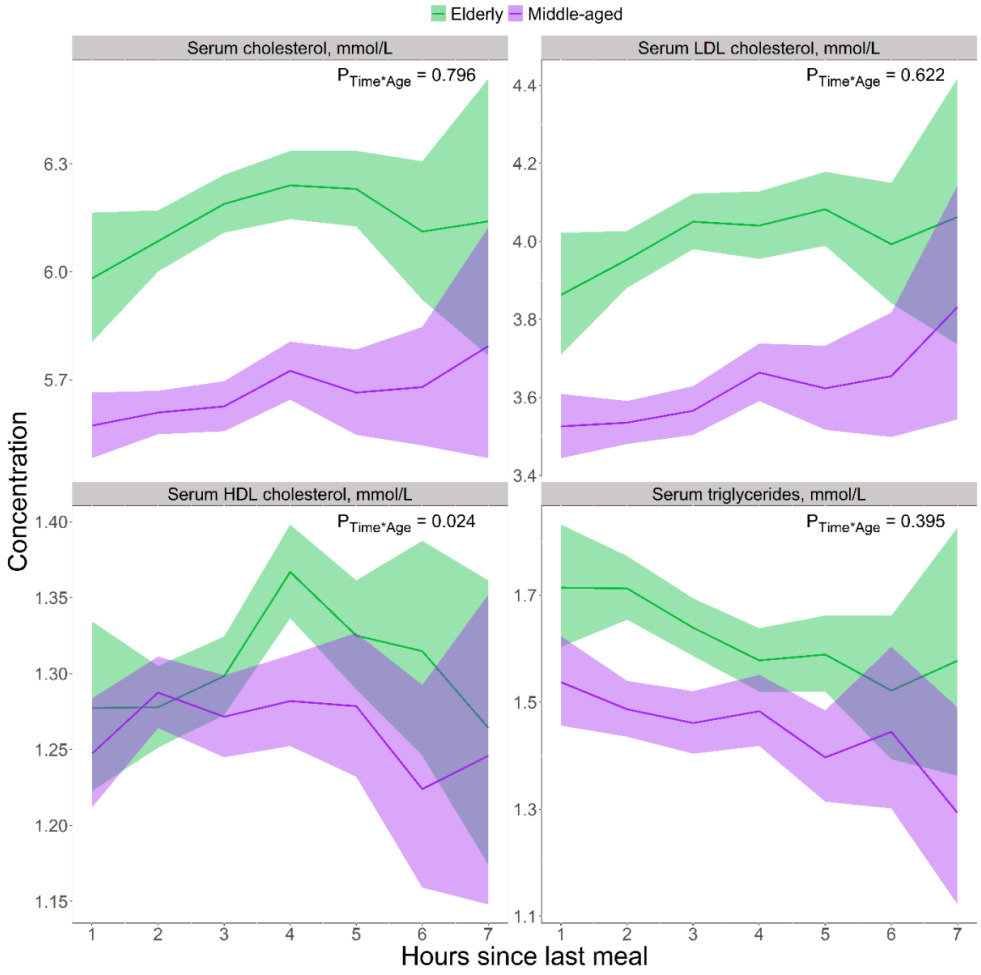
Supplementary Figure 2. The concentration of glucose as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represents the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



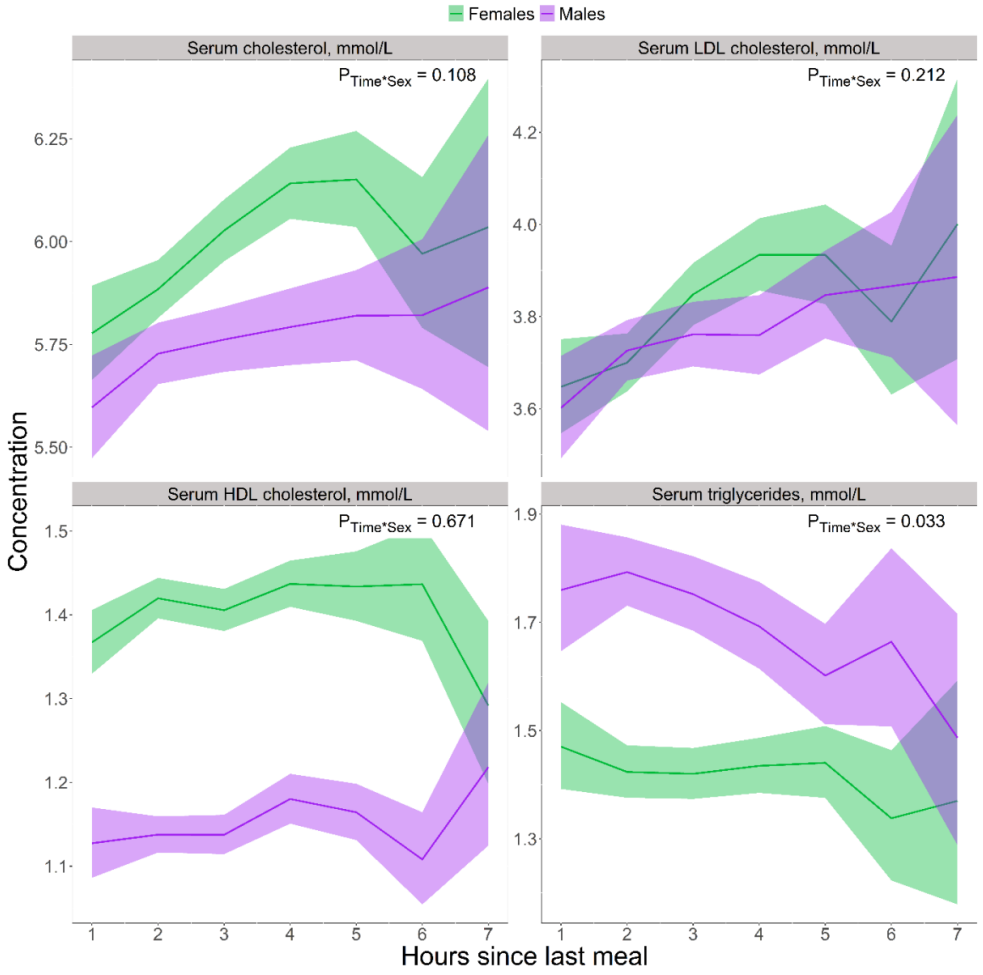
Supplementary Figure 3. The concentration of amino acids as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represents the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



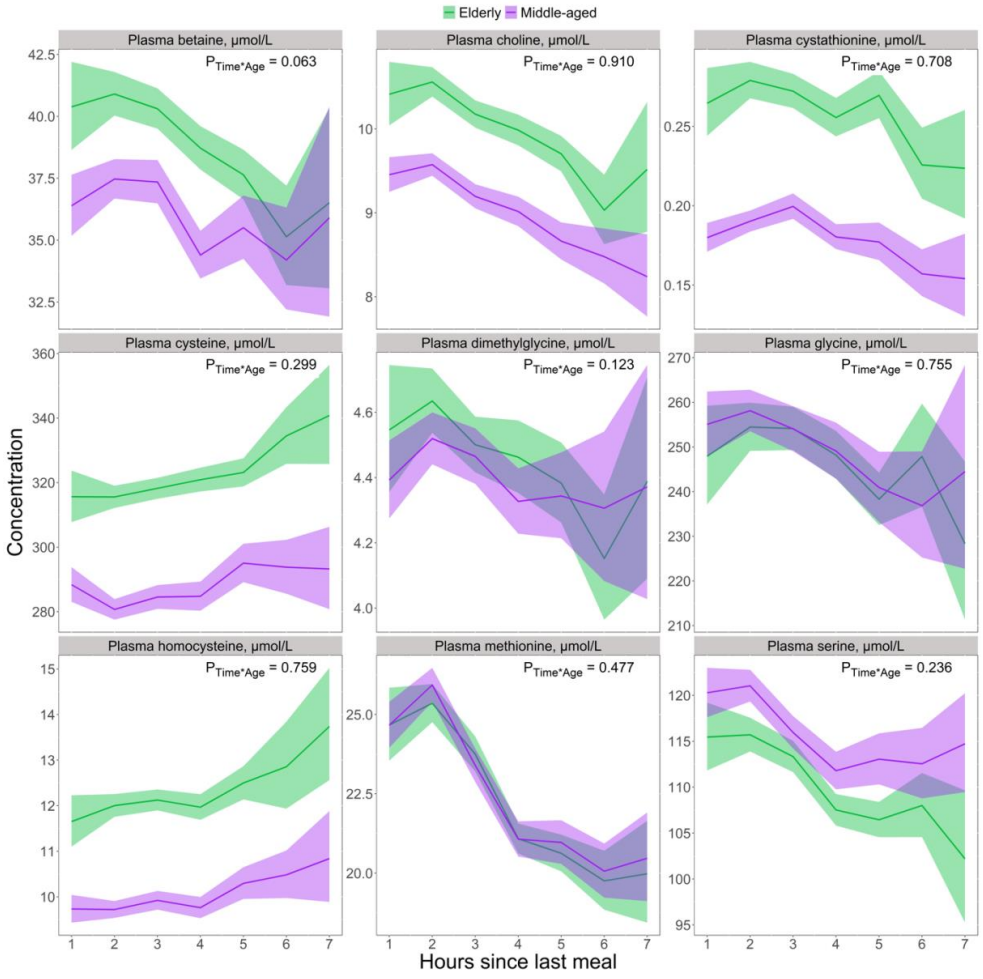
Supplementary Figure 4. The concentration of amino acids as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5



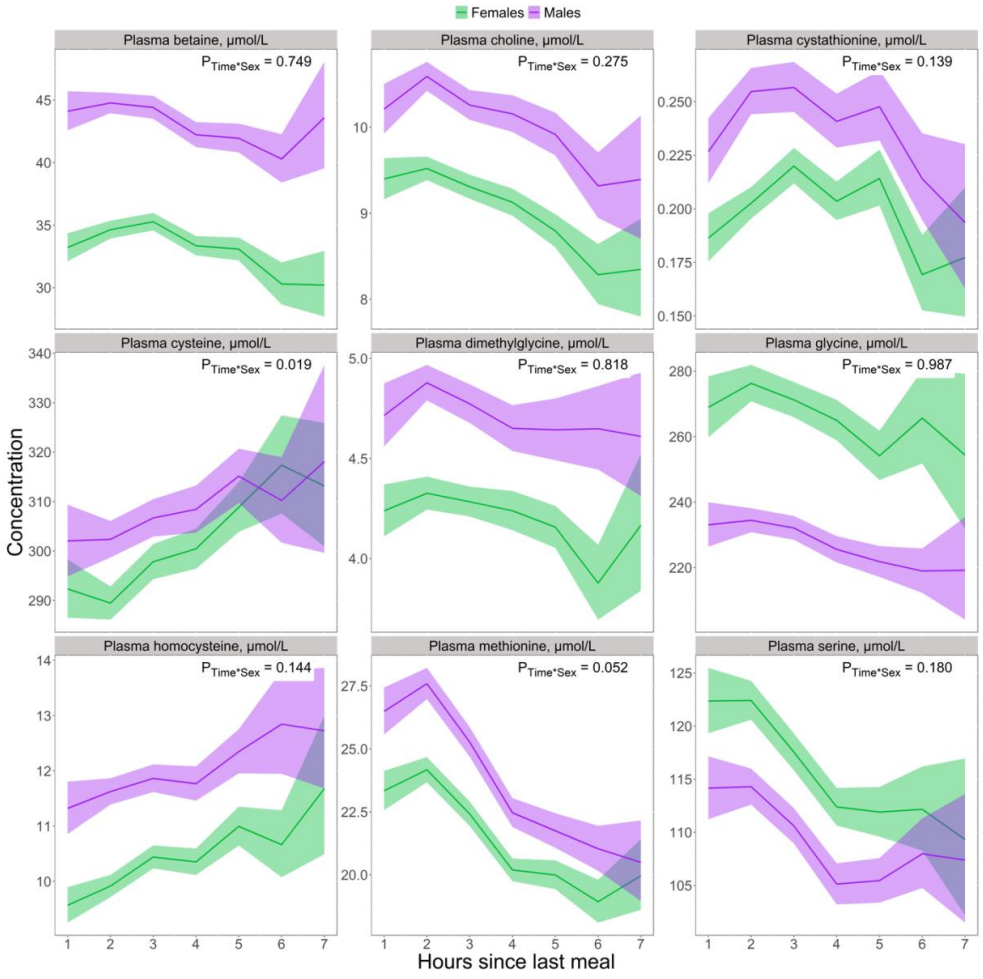
Supplementary Figure 5. The concentration of blood lipids as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5. **Abbreviations**: HDL, High density lipoprotein; LDL, low density lipoprotein.



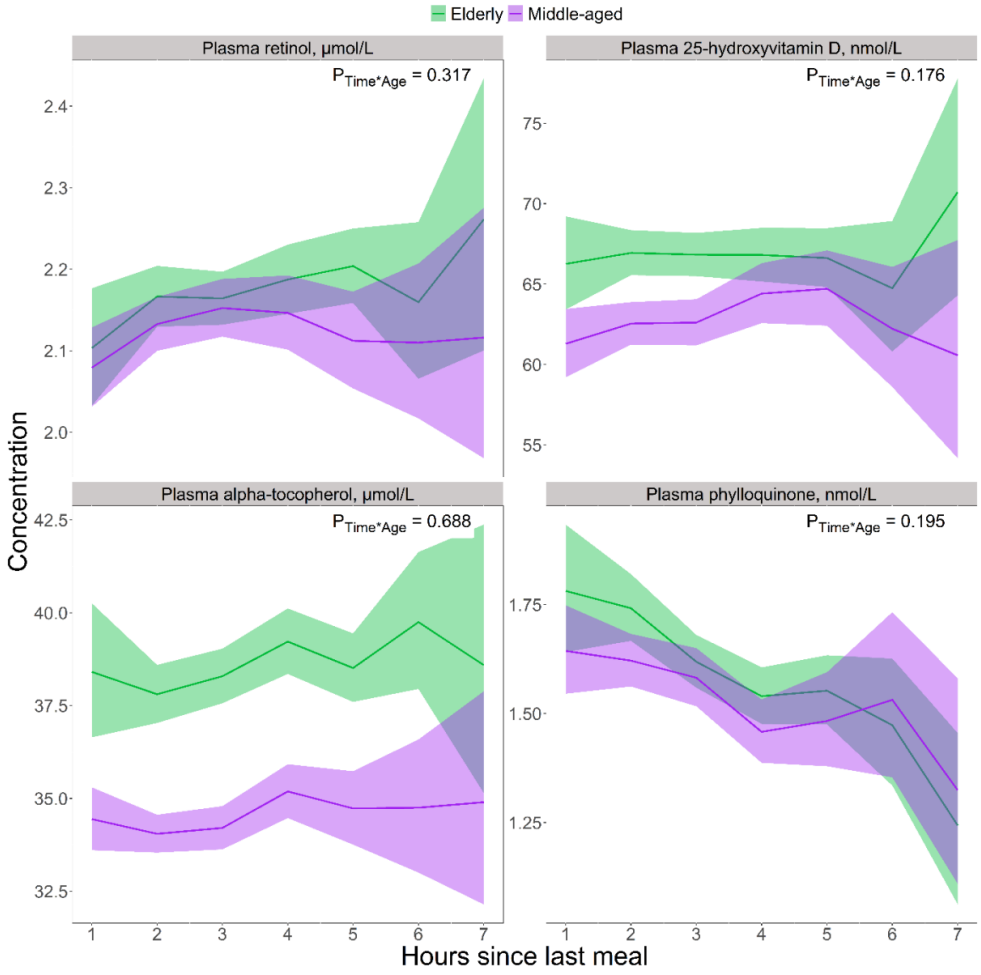
Supplementary Figure 6. The concentration of blood lipids as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5. **Abbreviations**: HDL, High density lipoprotein; LDL, low density lipoprotein



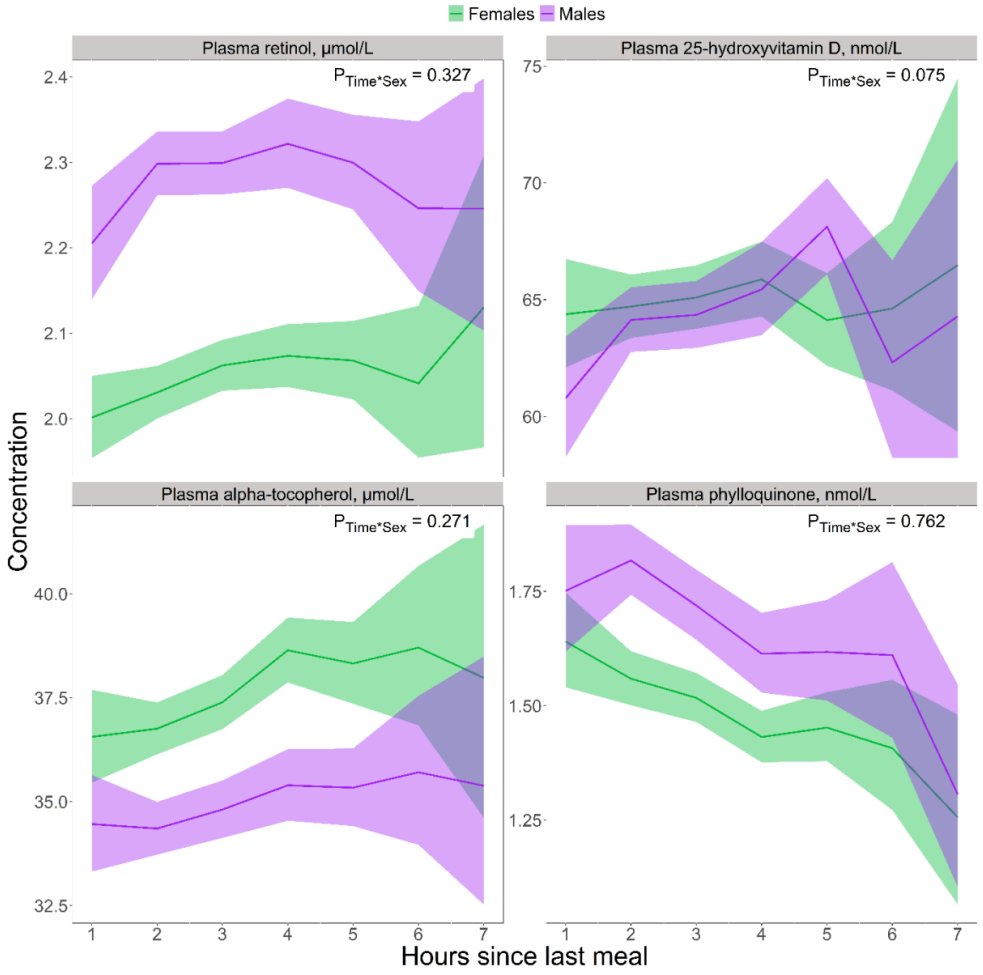
Supplementary Figure 7. The concentration of one-carbon metabolites as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



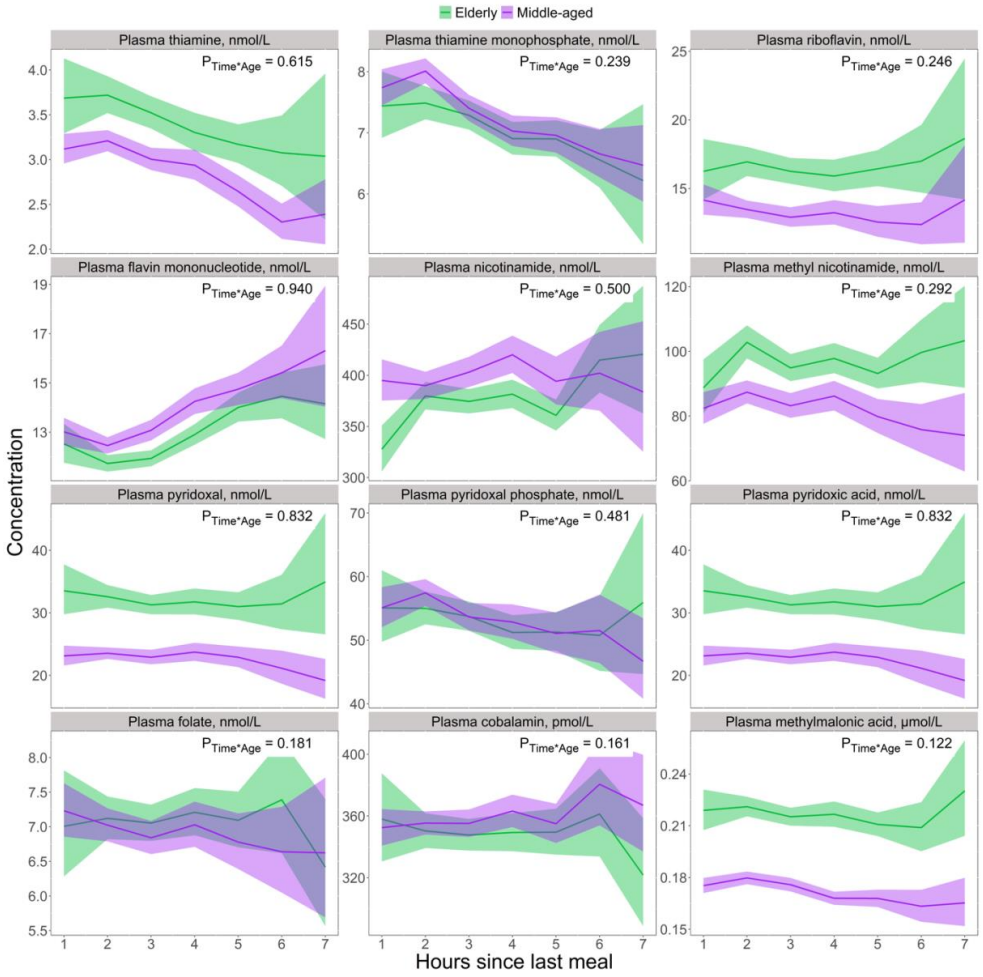
Supplementary Figure 8. The concentration of one-carbon metabolites as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



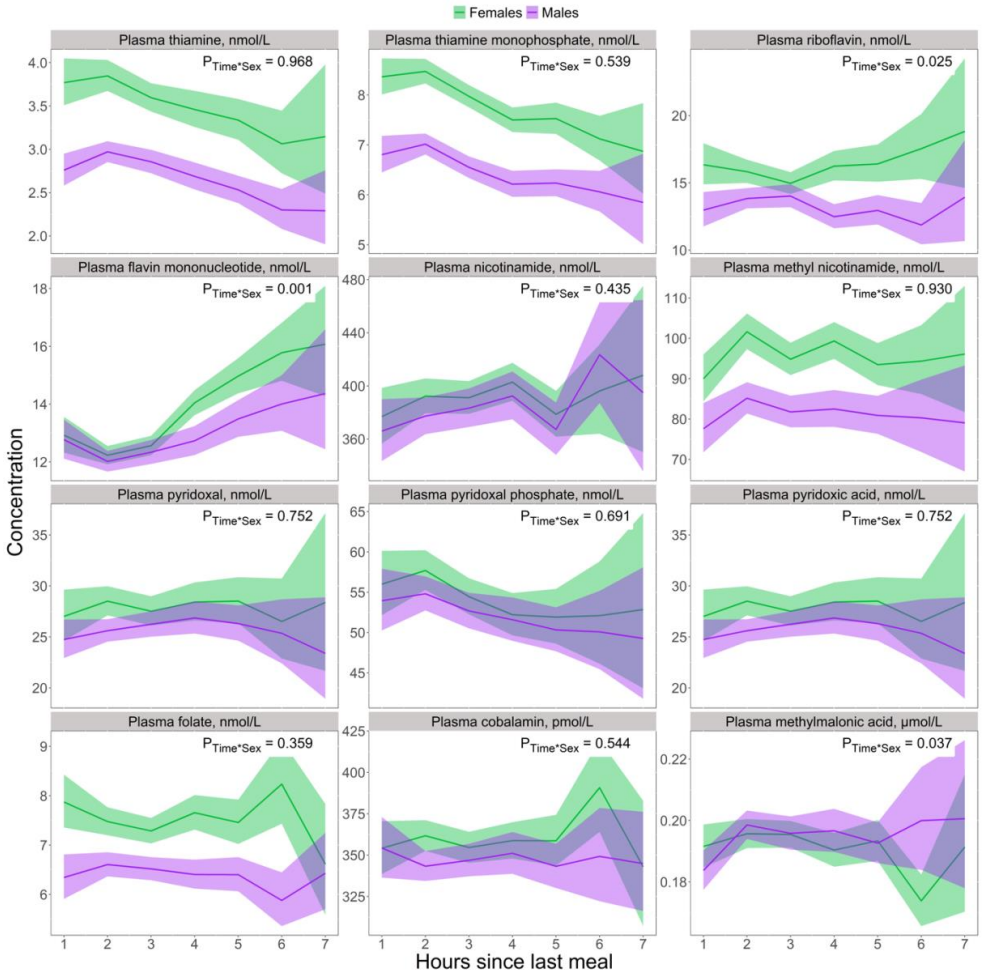
Supplementary Figure 9. The concentration of lipid-soluble vitamins as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represents the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



Supplementary Figure 10. The concentration of lipid-soluble vitamins as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



Supplementary Figure 11. The concentration of B-vitamin biomarkers as a function of time since the last meal in the middle-aged (n = 2960) and the elderly group (n = 2874) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.



Supplementary Figure 12. The concentration of B-vitamin biomarkers as a function of time since the last meal in males (n = 2541) and females (n = 3293) in the Hordaland Health Study 1997-1999. The solid line represents the geometric mean, while the colored shaded area represent the 95% geometric confidence intervals. Note that the origin of the y-axis ≠ 0, and the y-axes are scales to be compatible with the metabolite concentration ranges. An overview of the number of observations at each timepoint is provided in Supplementary Table 4 and 5.

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Time-resolved concentrations of serum amino acids, one-carbon metabolites and B-vitamin biomarkers during the postprandial and fasting state: the Postprandial Metabolism in Healthy Young Adults (PoMet) Study

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Abstract

Metabolomics has been utilised in epidemiological studies to investigate biomarkers of nutritional status and metabolism in relation to non-communicable diseases. However, little is known about the effect of prandial status on several biomarker concentrations. Therefore, the aim of this intervention study was to investigate the effect of a standardised breakfast meal followed by food abstinence for 24 h on serum concentrations of amino acids, one-carbon metabolites and B-vitamin biomarkers. Thirty-four healthy subjects (eighteen males and sixteen females) aged 20–30 years were served a breakfast meal (~500 kcal) after which they consumed only water for 24 h. Blood samples were drawn before and at thirteen standardised timepoints after the meal. Circulating concentrations of most amino acids and metabolites linked to one-carbon metabolism peaked within the first 3 h after the meal. The branched-chain amino acids steadily increased from 6 or 8 hours after the meal, while proline decreased in the same period. Homocysteine and cysteine concentrations immediately decreased after the meal but steadily increased from 3 and 4 hours until 24 h. FMN and riboflavin fluctuated immediately after the meal but increased from 6 h, while folate increased immediately after the meal and remained elevated during the 24 h. Our findings indicate that accurate reporting of time since last meal is crucial when investigating concentrations of certain amino acids and one-carbon metabolites. Our results suggest a need for caution when interpretating studies, which utilise such biomarkers, but do not strictly control for time since the last meal.

Keywords: **Postprandial Response: Fasting: Metabolism: Metabolites: Metabolomics: Biomarkers: Epidemiology**

The metabolome is dynamic and constantly changing in response to external stimuli. Dietary intake is one such external factor triggering notable metabolic and hormonal changes in the hours following food intake. For instance, postprandial concentrations of glucose and insulin change in response to the intake of specific food or nutrients[1,2]. Consequently, prandial status at the time of blood sampling is accounted for when defining the normal ranges of glucose and insulin[3]. The metabolome undergoes dynamic changes not only immediately after dietary intake but also during prolonged fasting. In 2011, Rubio-Aliaga and colleagues[4] reported an analysis of the 'human fasting metabolome'. In their study, ten healthy volunteers fasted for 36 h, and blood samples were taken at 12 and 36 h after the last meal. They observed that over 70 % of circulating amino acid concentrations changed during prolonged fasting, with notable decreases in methionine and tryptophan and increases in the branched-chain amino acids (BCAA). In 2012, Krug *et al.*[5] investigated metabolite changes in response to several metabolic challenges, including 36 h of fasting in fifteen healthy males. Like Rubio-Aliaga *et al.*, they reported that the BCAA and NEFA concentrations increased during 36 h of fasting. Teruya *et al.*[6] investigated a range of metabolites during 58 h of fasting in a

Abbreviations: BCAA, branched-chain amino acid; gSE, geometric standard error; ICC, intra-class correlation coefficient; PA, pyridoxic acid; TMP, thiamine monophosphate.

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small study of four participants. Similarly, they also reported increased concentrations of BCAA in addition to changes in butyrates, acylcarnitines, some co-enzymes and other amino acids.

Circulating metabolite concentrations are frequently investigated in epidemiological studies as predictors or mediators of health and disease outcomes. For instance, homocysteine concentrations have been extensively investigated as a risk factor for a variety of diseases including CVD[7] and dementia[8]. High concentrations of the BCAA are also reported as biomarkers for increased risk of diabetes[9]. However, there are several potential sources of measurement error that should be considered when evaluating blood biomarker concentrations in epidemiological studies. Such measurement errors are not limited to sample processing and laboratory analyses but may also arise from the within-person variability of the biomarker. As the concentrations of blood metabolites may fluctuate within subjects in response to dietary intake and prandial status, a single measurement of the biomarker may be a poor measure of the true aetiologic exposure[10]. To limit the impact of prandial status, it is common in epidemiological studies to apply a distinct cut-off to distinguish between the postprandial and the fasting state. This may be convenient, but the transition between the two states exists on a continuum with no clear cut-off for being in one state or the other. Further, the duration of the postprandial state is influenced by factors such as meal size and composition. Smaller meals high in simple carbohydrates are typically digested and absorbed more rapidly, resulting in a postprandial period of approximately 2–3 h. On the other hand, larger meals that are rich in fats can extend the postprandial period up to 8 h[11,12]. In the literature, some use 90 min[13], 2 h[14], 3 h[15], 4 h[16] or 6 h[17] to define the postprandial state. It is evident that the duration of the postprandial period is challenging to define precisely due to its variability, and that applying a cut-off at a certain time after a meal may not sufficiently account for the fluctuating nature of the metabolome in response to dietary intake and fasting.

Although previous studies have demonstrated metabolic changes linked to the postprandial and fasting states, most studies tend to commence blood sampling after overnight fast[4–6] or only during the first few hours after a meal[18]. Data on the metabolic changes during the adaption from the postprandial to the fasting state are missing. Therefore, the aim of this study was to investigate how serum concentrations of amino acids, one-carbon metabolites and B-vitamin biomarkers change during the 24 h after a standardised breakfast meal in healthy, young individuals.

Methods

Recruitment and pre-screening

Information about the study was spread through social media channels and posters in the nearby area of Bergen, Norway during the summer of 2021. Individuals who were interested in participating in the study were contacted and pre-screened over the phone, and individuals eligible for inclusion were invited to the main screening and to attend the study visit. Overall, the aim was to recruit young, healthy participants. The inclusion criteria were: (1) aged 20–30 years (born 1991–2001); (2) self-reported BMI 22–27 kg/m^2 at phone screening, while subjects were excluded if they (1) had experienced acute or chronic disease such as diabetes, thyroid diseases, cancer, CVD or inflammatory bowel disease during the last 3 years; (2) had celiac disease or other food allergies interfering with the standardised breakfast meal; (3) used any prescription medications except for contraceptives; (4) smoked or used other nicotine-containing products such as 'snuff' regularly; (5) had been pregnant or breastfed the last 3 months before study visit and (6) had experienced weight change > 5 % during the last 3 months before the study visit. The inclusion and exclusion criteria for participation in the study are summarised in Table 1.

Table 1. Inclusion and exclusion criteria for participation in the Postprandial Metabolism Study

Inclusion criteria	Exclusion criteria
• Aged 20–30 years (birth years 1991–2001) • Self-reported BMI 22–27 kg/m^2 at phone screening	• Acute or chronic disease such as diabetes, thyroid diseases, cancer, CV or inflammatory bowel disease during the last 3 years • Celiac disease or other food allergies interfering with the standardised breakfast meal • Use of any prescription medications except contraceptives • Smoking or regular use of other nicotine-containing products, such as 'snuff' • Pregnancy of breast-feeding the last 3 months before the study visit • Significant weight change (> 5 %) during the last 3 months before the study visit

Instructions before the study visit

To standardise physiological and metabolic conditions, all individuals were instructed to (1) not use dietary supplements the last 7 d before the visit; (2) abstain from smoking and use of nicotine-containing products such as 'snuff' the last 7 d before the visit; (3) abstain from alcohol and avoid any strenuous activity the last 24 h before the visit; (4) consume an evening meal consisting of three slices of bread with cheese and jam, and a glass of juice at 20.00 the evening before the study visit; (5) not consume anything other than water after the evening meal before the study visit and (6) drink enough water and to stay hydrated to facilitate blood sampling and the insertion of a venous catheter.

Study visits

The course of the study visits in this intervention study is illustrated in Fig. 1. The study was conducted at the Research Unit for Health Surveys, the University of Bergen, Norway. On the morning (between 07.30 and 08.00 hours) at the attendance of the study visit, all individuals had their height and body weight measured to calculate their BMI for screening purposes. The measurements were conducted by the same researcher at all study visits to ensure similar measurements. Height was measured to the nearest 0·1 cm using a Seca 217 stadiometer,

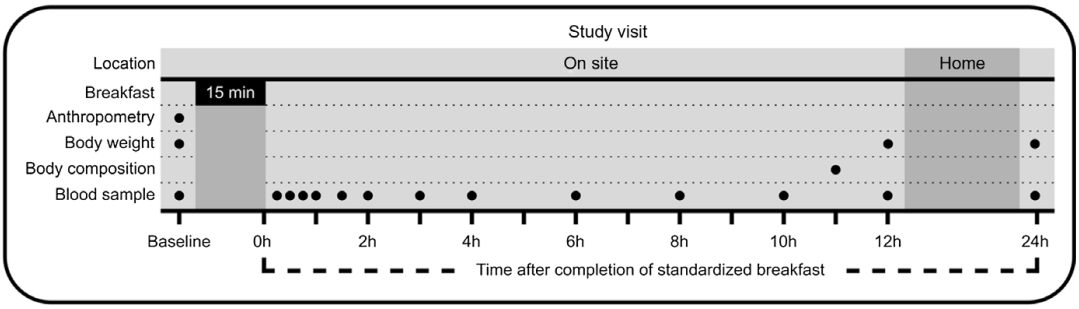

Figure 1. Sampling times in the Postprandial Metabolism Study.

with individuals standing without shoes and in light clothing, feet gathered and the head positioned in the Frankfurt horizontal plane. Body weight was measured and rounded to the nearest 0·1 kg using a Seca 877 flat scale, measured without shoes and in light clothing. Due to variation between scales (home *v.* study centre), clothing and hydration status when measuring body weight, some deviations from the BMI criteria (self-reported BMI between 22 and 27 kg/m^2) at study entry were accepted. Participants who fulfilled all inclusion and exclusion criteria according to Table 1 were included in the study. Adherence to the instructions before the study visit was controlled by self-reported questionnaires, but participants were not excluded if they deviated from the instructions.

Blood sampling and preanalytical handling of blood samples

A venous catheter was placed in the elbow cavity. Ten minutes after the insertion of the catheter, a venous blood sample and capillary blood glucose (Hemocue® Glucose 201 RT Analyzer) were taken. Thereafter, the standardised breakfast meal was served. After consumption of the meal, a total of thirteen blood samples were drawn as shown in Fig. 1. Blood samples were drawn particularly frequently during the first 4 h (timepoints 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 3 h and 4 h after the meal), and then every other hour until 12 h after the meal. After the 12-h blood sample, the participants left the study centre overnight and came back the next morning for the last blood sample taken 24 h after the breakfast meal (Fig. 1). Capillary blood glucose was also measured at each timepoint before the venous blood sampling. After the 10-h blood sample, the venous catheter was removed to facilitate the body composition analysis. Therefore, the 12- and 24-h blood samples were taken as normal venous blood samples. At each timepoint, a total of 11·5 ml of blood was drawn and distributed into serum tubes (8·5 ml, BD Vacutainer® SST™ II *Advance*; Becton, Dickinson, and Company) and EDTA tubes (3 ml, Vacuette® K2EDTA). At baseline and at the 24-h timepoint, an additional 6 ml and 3 ml EDTA blood was collected, respectively, for measurement of haematology and safety biomarkers (aminotransferases, creatinine, C-reactive protein, erythrocytes, gamma-glutamyltransferase, Hb, hBA1c, mean corpuscular Hb, mean corpuscular volume, mean platelet volume, thrombocytes, thyroid stimulating hormone and 25-hydroxyvitamin D).

After the blood sampling, the serum tubes were stored at room temperature for 30–60 min and then centrifuged at 2200 ×***g*** for 10 min at 20°C. EDTA tubes were centrifuged within 15 min after collection, at 2200×***g*** for 10 min at 4°C. Three aliquots of serum and one aliquot of plasma per timepoint were temporarily stored in a freezer at −20°C and transferred to −80°C at the end of the day. Additionally, one aliquot of serum from each time point was stored in the refrigerator at +4°C and transported to the laboratory daily, together with the additional EDTA blood collected at baseline and the 24 h timepoint.

Breakfast meal

The breakfast meal consisted of wholegrain wheat bread with butter, strawberry jam, low-fat cheese (16 % fat), cucumber and a glass of orange juice. The amounts and nutrient composition of the breakfast meal are given in Table 2. The breakfast meal was composed to mimic a normal Norwegian breakfast and provided 20–24 % of the daily energy needs, which are estimated to be 2600 kcals and 2150 kcal per day for inactive males and females, respectively[19]. All participants were instructed to consume the breakfast in precisely 15 min, and the minute the last bite was consumed was set to timepoint zero. After the breakfast meal was consumed, the participants were instructed not to consume anything other than water (no chewing gum, sparkling water, diet soda, etc.) for the next 24 h.

Body composition analysis

After the 10-h blood sample, body composition was analysed using a BodPod (COSMED, version 5.4.6). To standardise measurements, the analysis was conducted by the same researcher at all study visits, and all participants were instructed not to consume any water for the last 2 h before the measurement. The analysis was carried out with participants wearing swimwear or underwear of synthetic material and a swimming cap, and wearing no jewellery or piercings.

Laboratory analyses

The measurement of capillary glucose was performed using a handheld device (Hemocue® Glucose 201 RT Analyzer). All amino acids and one-carbon metabolites were analysed in serum at Bevital AS. Alanine, asparagine, aspartic acid,

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Table 2. The breakfast meal in the Postprandial Metabolism Study*

Food item	Wheat bred	Butter	Low-fat cheese	Strawberry jam	Orange juice	Cucumber	Total
Grams	90	15	40	20	200	36	401 g
Energy (kcal)	213	81	107	26	82	4	513 kcal
Macronutrients							
Fat (g)	4·7	9	6·4	0	0·4	0	20·5 g (35·9 E%)
Carbohydrate (g)	34·5	0·1	0	6·2	18·2	0·4	59·4 g (46·3 E%)
Dietary fibre (g)	4·0	0	0	0·3	0·2	0·3	4·8 g (1·9 E%)
Protein (g)	6·2	0·1	12·4	0·1	1·4	0·3	20·5 g (16·0 E%)
B-vitamins†							
Thiamine (mg)	0·27	0	0·1	0	0·16	0·01	0·54 mg
Riboflavin (mg)	0·07	0	0·1	0	0·04	0·01	0·22 mg
Niacin (mg)	2	0	0·8	0	0·4	0·1	3·3 mg
Vitamin B_6 (mg)	0·09	0	0·02	0·01	0·1	0·01	0·23 mg
Folate (µg)	29	0	17	3	56	4	109 µg
Vitamin B_{12} (µg)	0	0	1	0	0	0	1 µg

* The same breakfast was provided to all participants irrespective of body weight or sex.
† The B-vitamins were estimated using 'Matvaretabellen' (www.matvaretabellen.no), a tool developed by the Norwegian Food Safety Authority and the Norwegian Directorate of Health.

cystathionine, cysteine, glutamic acid, glutamine, glycine, histidine, total homocysteine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, sarcosine, serine, threonine, tryptophan, tyrosine and valine were analysed using a gas-chromatography mass spectrometry (GS-MS/MS), while arginine, betaine, choline, dimethylglycine and methionine sulfoxide were analysed using a liquid-chromatography mass spectrometry (LC-MS/MS). Among the B-vitamin biomarkers, serum cobalamin and total serum folate (i.e. the sum of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and pteroylglutamic acid) were analysed at the Department of Medical Biochemistry and Pharmacology at Haukeland University Hospital, Bergen, Norway (certified NS-EN ISO 15189:2012) using immunoassay. FMN, N1´-methylnicotinamide, nicotinamide, pyridoxal, pyridoxal-5-phosphate, 4´-pyridoxic acid, riboflavin, thiamine and thiamine monophosphate (TMP) were analysed at Bevital AS using an LC-MS/MS, while methylmalonic acid was analysed using a GC-MS/MS. An overview of the metabolites and safety markers measured, and their analytical methods are found in online Supplementary Table 1.

Quality assurance

Standardised operating procedures were developed and followed throughout the study to ensure accurate and similar measurements. Qualified personnel conducted the blood sampling, and efforts were made to have the same staff member carry out data collection on the same participant to avoid systematic differences in data collection.

Statistical analyses

All statistical analyses were performed using R version 4.1.3 (R Foundation for Statistical Computing, https://www.r-project.org/) and the packages within the *tidyverse* and *irrICC*.

Negative values are not possible with biological data. Further, most biomarkers are skewed with a longer tail towards higher values. Therefore, all metabolite concentrations were log-transformed before statistical analysis and described using the back-transformed gMean and gSD as recommended[20,21].

Descriptive statistics are supplemented with ranges (min–max). Inferential statistics are accompanied by 95 % geometric compatibility (confidence) intervals (gCI) as a measure of uncertainty, calculated using the geometric standard error (gSE) and formulas 1–3:

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

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

$$95\%\ \mathrm{CI\ upper\ limit} = \mathrm{gMean} \times \mathrm{gSE}^{1.96} \quad (3)$$

The main objective is presented visually, by plotting the raw metabolite concentrations as a function of time, with the mean time-course indicated by superimposing the geometric mean concentrations (95 % gCI) on top of the individual data. Relative changes in metabolite concentrations were calculated for each individual, with each pre-breakfast blood sample utilised as an individual reference value. These individual percentage changes were subsequently combined to calculate the gMean percentage change across the study cohort. To evaluate the degree to which the different biomarkers are affected during the postprandial period, the within-person reproducibility was quantified by calculating intra-class correlation coefficients (ICC) on log-transformed data. The ICC were calculated on the basis of a two-way random-effects model for absolute agreement, using the *irrICC* package and the function *icc2.nointer.fn()*[22].

Sample size calculation

The sample size calculation was performed using an accuracy-in-parameter-estimation approach, as recommended when the main purpose is to accurately estimate the parameters of interest[20,21]. For the main analysis, we aimed to achieve a multiplicative margin-of-error (gSE1·96) < 1·10, corresponding to a gSE < 1·05, for at least 80 % of the measurements. Using freely available data on 132 metabolites across fifty-six time-points (7392 estimates) across different metabolic challenges

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from the HuMet study[5] (available from http://metabolomics.helmholtz-muenchen.de/humet/), the observed median (80th percentile) gSD was 1·24 (1·32). Rearranging equation 1 above, and solving for n with a gSD = 1·32, we needed a sample size of 32 to achieve the desired precision level. Precision curves as a function of sample size are provided in online Supplementary Fig. 1(a). The expected distribution of multiplicative margin-of-errors with a sample size of 32 is illustrated in online Supplementary Fig. 1(b), based on repeated resampling with replacement from HuMet (50 replications, 369 600 simulated estimates). We expected a dropout of up to 10 % due to adverse events following fasting blood sampling or difficulties drawing blood from a venous catheter. Therefore, to achieve our goal of collecting complete data for thirty-two participants, we aimed to recruit a total of thirty-six participants (eighteen males and eighteen females).

Ethics and safety

The study was registered at ClinicalTrials.gov (NCT number 04989478). The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by The Regional Committee for Health Research Ethics (REK 236654). Written informed consent was obtained from all subjects. Participants received the consent form by email before the study visit to allow adequate time to read and understand the protocol and to familiarise themselves with the risk, burdens and benefits of participation in the study. In addition, one of the researchers went through the consent form in oral with the participants, and participants were explicitly allowed to ask additional questions before signing the consent form. Participants who communicated great discomfort, either orally or by body language (syncope, etc.), during the study visit were excluded from the study. Participants were also excluded if there were difficulties with blood sampling from the venous catheter (n 2).

Results

Study participants

A flow chart depicting the inclusion of participants is illustrated in Fig. 2. A total of forty-nine individuals completed the web-based questionnaire and were contacted by phone for a pre-screening. Of these, forty-seven individuals fulfilled the inclusion and exclusion criteria. For three of the subjects, an agreed date for participation could not be found, while eight subjects withdrew before the study visit. Therefore, a total of thirty-six individuals were included in the study. Two participants (both female) withdrew from the study right after the breakfast meal due to difficulties with blood sampling and were excluded from all analyses. Additionally, one participant completed the first 2 h (eight blood collection timepoints) before withdrawing due to difficulties with blood sampling. This participant was included in the analyses. In total, thirty-three participants completed the whole study, while data from thirty-four participants are included in the analyses.

Participant characteristics

Complete participant characteristics are provided in Table 3. A total of eighteen males and sixteen females were included in the analyses. The age ranged from 20 to 30 years old, and thirty-three out of thirty-four participants were of Caucasian ethnicity, with one participant of Asian ethnicity. Of the female participants, thirteen participants (81 %) used some form of contraceptive, with the most common being combined oral contraceptives (n 6), followed by contraceptive implant (n 3), hormonal intra-uterine device (n 2), copper intra-uterine device (n 1) and progestin-only pill (n 1). On average, male participants had slightly higher BMI, waist circumference and RMR but a lower percentage of fat mass compared with female participants. All participants were considered healthy as evaluated by self-reported questionnaires and routine clinical measurements (online Supplementary Table 1).

Change in metabolite concentrations during 24 h after the meal

The relative change in concentrations of the metabolites investigated, accompanied with the ICC, is presented in Table 4 (total population) and Table 5 (males and females separately). Further, the absolute metabolite concentrations at all timepoints are presented in online Supplementary Table 2 (total population) and online Supplementary Table 3 (males and females separately). Figures illustrating the relative change in biomarker concentrations for the total population can be found in online Supplementary Figures 2–5.

Glucose and insulin

Glucose concentrations (Fig. 3(a)) increased immediately after the meal, peaking at 15 min (+41·8 % increase), before returning to baseline values at 90 min after the meal. Concentrations decreased slightly thereafter, falling to their lowest values at 24 h (–14·0 % decrease from baseline levels). For insulin (Fig. 3(b)), we observed a similar pattern, with concentrations peaking at 30 min (+827 % increase) and thereafter decreasing, falling to their lowest values at 10 h (–56·4 % decrease from baseline values). The results were comparable between the sexes (Table 5).

Amino acids

We observed a consistent pattern for nearly all amino acids, with increased concentrations right after the meal. This pattern was observed for alanine, arginine, asparagine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, tyrosine and valine (Fig. 4(a)–(p)). The largest relative increases in serum concentrations were observed for proline (Fig. 4(l), +55·5 % increase from baseline values), alanine (Fig. 4(a), +42·4 %) and tyrosine (Fig. 4(o), +29·2 %). The concentrations of the BCAA (Fig. 4(h), (i), (p)) increased from 6 (isoleucine and leucine) or 8 (valine) h to 24 h, with peak concentrations observed at 24 h after the meal (isoleucine: +28·0 %, leucine: +37·2 %, valine: +14·4 % increase from baseline values). In contrast, the concentrations of proline (Fig. 4(l)) decreased from the 6- to the 24-h timepoint, reaching the lowest values at 24 h (–19·3 % decrease from baseline values). The levels of aspartic acid (Fig. 4(d)) fluctuated

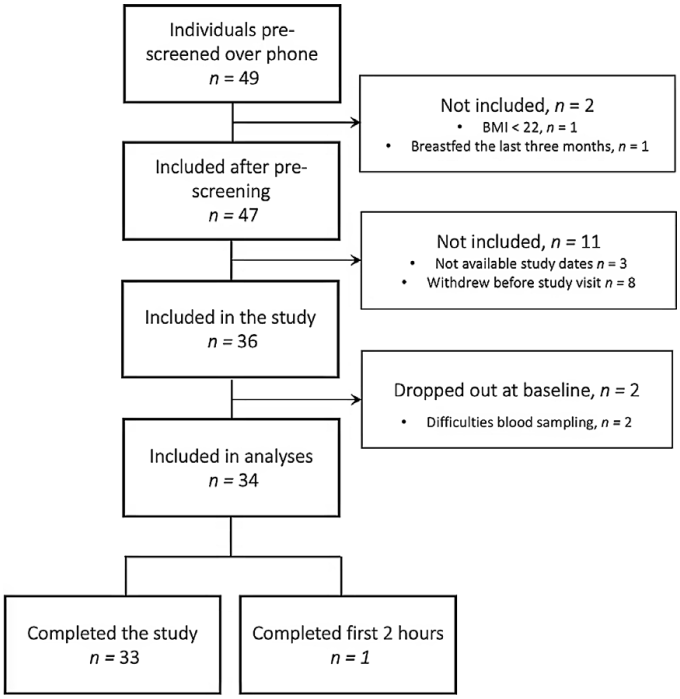

Figure 2. Flow chart of the inclusion process for participants in the Postprandial Metabolism Study.

Table 3. The main characteristics of the participants (*n* 34) included in the Postprandial Metabolism Study

	Total population *n* 34			Males *n* 18			Females *n* 16		
	gMean	gSD	Min–max	gMean	gSD	Min–max	gMean	gSD	Min–max
Age, years	25·3	1·13	20–30	26·1	1·12	20–30	24·5	1·13	20–29
Caucasian ethnicity									
n	33		–	18		–	15		–
%	97 %			100 %			93·7 %		
Height, cm	176·3	1·06	152·5–194·7	184·2	1·03	173·2–194·7	167·8	1·05	152·5–180·9
Weight, kg	73·3	1·15	57·2–98·3	80·9	1·10	64·7–98·3	65·2	1·09	57·2–76·6
BMI, kg/m^2	23·5	1·07	202–26·9	23·8	1·07	21·2–26·9	23·1	1·07	20·2–25·5
Waist circumference, cm	81·5	1·11	67·3–100	88·1	1·08	80–100	74·6	1·06	67·3–80·0
Fat mass percentage*	22·4	1·43	10·5–41·9	18·3	1·41	10·5–39·1	28·6	1·19	21·6–41·9
RMR*	1508	1·21	1053–2200	1740	1·10	1433–2200	1269	1·12	1053–1552
Use of contraceptives									
n	13		–	–		–	13		–
%	38·2 %						81·3 %		

* Estimated using BodPod (COSMED).

immediately after the meal but stabilised around baseline levels at 3 h, while the levels of glutamic acid (Fig. 4(e)) fluctuated during the first hour after the meal before remaining decreased from 90 min onwards, with the lowest values observed at 24 h (–15·6 % decrease from baseline values). For glutamine (Fig. 4(f)), the concentrations appeared to be slightly elevated during the first 12 h, before returning to baseline values 24 h after the meal. The ICC for the amino acids ranged from 0·49 to 0·56. Male participants had, on average, slightly higher concentrations of nearly all amino acids except for aspartic acid, in which females had higher concentrations. However, the relative changes in concentrations after the meal were comparable between sexes for all amino acids (Table 5).

One-carbon metabolites

For the one-carbon metabolites, we observed similar results to those observed for the amino acids; the concentrations of

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Table 4. The relative change in metabolite concentrations (% change from reference values) after consumption of a standardized meal in healthy subjects in the Postprandial Metabolism Study

		Time after meal												
Serum biomarker	**Ref***	15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours	ICC
n	34	34	34	34	34	34	34	33	33	33	33	33	33	33	-
Glucose and insulin															
Glucose†	5·27 mmol/L	41·8	40·9	20·0	8·52	−0·13	−4·97	−1·58	−2·19	−6·54	−11·5	−11·0	−13·4	−14·0	0·39
Insulin	4·45 mIU/L	746	827	631	502	296	173	70·0	−12·3	−44·2	−52·4	−56·4	−54·4	−53·9	0·20
Amino acids															
Alanine	331 µmol/L	25·8	36·2	41·3	42·4	40·9	34·7	21·0	8·99	−5·89	−10·3	−12·5	−11·2	1·87	0·49
Arginine	85·8 µmol/L	21·2	15·3	16·8	14·4	18·1	19·3	8·41	−1·19	−4·64	−2·41	−1·94	2·22	0·07	0·51
Asparagine	66·5 µmol/L	31·4	30·6	29·1	27·6	26·2	21·9	9·45	−0·30	−5·42	−3·54	−5·47	−5·87	−6·22	0·49
Aspartic acid	17·7 µmol/L	−0·93	−0·28	−1·12	−0·32	−0·25	−0·45	2·51	2·49	1·43	3·36	4·92	3·55	1·45	0·56
Glutamic acid	43·1 µmol/L	−6·98	5·07	6·17	6·76	−5·60	−7·50	−6·51	−9·37	−14·2	−13·2	−10·9	−14·1	−15·6	0·53
Glutamine	506 µmol/L	5·49	5·32	4·78	4·97	8·44	9·62	8·41	6·99	6·18	7·30	6·93	4·15	0·45	0·51
Histidine	80·1 µmol/L	8·40	10·17	9·82	9·63	13·1	15·5	10·8	3·82	−1·11	−0·68	−0·44	0·90	5·80	0·52
Isoleucine	64·6 µmol/L	17·4	17·7	18·2	18·9	24·6	28·2	17·8	3·71	0·20	6·38	10·2	14·6	28·0	0·51
Leucine	128 µmol/L	14·6	14·7	15·2	15·8	20·6	23·8	15·8	5·20	3·82	9·83	14·1	16·6	37·2	0·50
Lysine	157 µmol/L	11·9	12·1	12·0	12·7	17·9	21·0	15·1	4·80	−2·49	−0·35	1·37	3·25	9·11	0·50
Phenylalanine	70·6 µmol/L	10·4	13·0	15·4	18·0	21·5	21·3	11·3	0·40	−3·80	1·93	3·80	4·76	5·01	0·50
Proline	179 µmol/L	30·6	38·3	45·6	51·2	54·7	55·5	41·2	24·8	7·75	0·08	−5·67	−10·59	−19·3	0·49
Threonine	132 µmol/L	15·3	15·7	14·0	13·3	13·2	11·4	4·20	−4·62	−10·1	−10·9	−12·6	−15·2	−14·5	0·51
Tryptophan	71·3 µmol/L	4·27	2·00	1·33	1·31	2·03	2·33	−4·08	−13·1	−18·2	−12·0	−8·37	−3·75	−11·0	0·50
Tyrosine	62·4 µmol/L	8·85	11·4	13·6	16·9	24·0	29·2	23·4	8·31	−9·12	−11·4	−13·2	−13·0	−8·98	0·51
Valine	250 µmol/L	6·79	8·04	7·88	7·81	10·7	13·7	11·7	6·27	2·17	1·56	1·77	2·78	14·4	0·50
One-carbon metabolites															
Betaine	33·0 µmol/L	10·9	15·6	18·4	20·4	25·1	24·9	14·9	10·2	3·80	3·40	−1·73	−3·81	−8·40	0·61
Choline	9·19 µmol/L	13·7	10·6	8·35	7·60	4·29	4·71	3·76	1·78	−0·09	5·31	−2·52	−7·24	−5·21	0·54
Cystathionine	0·18 µmol/L	−0·64	4·24	9·67	12·7	22·0	33·2	38·3	24·2	−7·52	−23·4	−28·2	−29·0	−24·6	0·64
Cysteine	251 µmol/L	−0·62	−2·66	−3·48	−3·60	−4·29	−4·81	−5·25	−3·56	0·36	5·13	7·61	9·59	11·6	0·50
DMG	3·45 µmol/L	1·57	2·32	2·24	5·37	11·9	11·6	10·0	4·88	−3·72	−4·36	4·48	5·70	8·16	0·82
Glycine	257 µmol/L	6·48	5·69	4·56	4·16	6·19	5·41	0·35	−2·29	−4·84	−3·79	−3·8	−4·69	−3·24	0·52
tHcy	8·16 µmol/L	−1·97	−3·33	−3·99	−3·30	−3·44	−3·00	−0·84	0·20	1·58	5·11	7·55	8·46	11·4	0·64
Methionine	29·3 µmol/L	15·7	16·7	18·7	20·8	27·0	29·2	14·0	−4·57	−14·0	−9·31	−8·05	−7·34	−4·69	0·49
MetSo	0·78 µmol/L	14·9	21·0	42·5	52·2	68·3	71·7	65·7	24·4	−0·82	−11·5	−17·5	−10·4	−15·5	0·25
Sarcosine	1·35 µmol/L	15·7	17·6	18·9	20·7	24·3	25·6	16·4	3·71	−9·10	−14·4	−18·5	−21·8	−30·0	0·69
Serine	131 µmol/L	14·1	15·6	14·5	13·8	12·7	12·1	6·42	1·07	−1·40	1·04	1·35	1·36	−0·44	0·50
B-vitamin markers															
Cobalamin	328 pmol/L	−1·92	−2·37	−2·65	−1·54	−2·26	−3·56	−1·36	0·09	0·53	2·45	5·13	5·73	13·5	0·55
FMN	8·07 nmol/L	−19·3	−24·9	−29·1	−30·0	−26·5	−22·3	−9·44	6·16	32·1	42·9	48·5	55·9	89·9	0·53
Folate	13·8 nmol/L	11·0	13·0	12·6	13·0	7·88	4·28	2·10	4·16	16·8	21·4	28·8	30·6	56·8	0·57
mNAM	106 nmol/L	20·7	17·7	10·4	8·83	10·2	7·99	−0·36	−0·46	5·92	6·68	−8·82	−28·0	1·37	0·59
MMA	0·14 µmol/L	1·33	0·15	1·08	2·09	3·71	5·92	4·14	−2·9	−12·9	−16·0	−18·4	−16·6	−12·0	0·63
Nicotinamide	203 nmol/L	38·0	24·1	6·40	−5·76	−13·1	−18·5	−22·9	−11·7	−8·12	−8·84	−17·1	−32·9	−11·3	0·54
Pyridoxal	17·1 nmol/L	10·8	13·1	13·7	13·0	7·64	6·78	−0·44	−3·75	−4·81	−5·61	−5·26	−5·39	−8·31	0·59
PLP	54·5 nmol/L	−3·05	−5·27	−7·62	−8·86	−10·5	−11·3	−10·9	−10·9	−12·6	−14·3	−17·4	−19·6	−14·9	0·59
4-pyridoxic acid	18·9 nmol/L	−3·08	−8·54	−18·3	−19·4	−24·6	−26·7	−24·8	−22·4	−21·6	−24·9	−24·5	−26·6	3·33	0·55
Riboflavin	13·5 nmol/L	−0·68	1·21	2·59	3·02	−0·42	−1·07	−4·39	−3·02	3·03	1·81	11·3	13·1	33·8	0·78
Thiamine	7·34 nmol/L	19·5	23·8	21·1	19·3	14·8	8·99	5·43	3·64	−1·13	−2·09	−3·11	−10·3	−19·8	0·60
TMP	3·05 nmol/L	11·4	4·79	4·65	−0·91	−3·85	−4·30	7·95	14·7	13·9	3·54	−3·05	−6·27	−12·5	0·88

ICC, Intraclass correlation coefficient; DMG, Dimethylglycine; FMN, Flavin mononucleotide; MetSo, Methionine sulfoxide; mNAM, N^1-methyl nicotinamide; MMA, Methylmalonic acid; PLP, Pyridoxal-5´-phosphate; tHcy, Total homocysteine; TMP, Thiamine monophosphate.

*Reference values are reported as geometric means. The reference values were measured around 8 a.m., 12 hours after a standard evening meal.

† Capillary glucose.

Table 5. The relative change in metabolite concentrations (% change from reference values) after consumption of a standardized meal in males ($n = 18$) and females ($n = 16$) subjects in the Postprandial Metabolism Study

		Time after meal														
Serum biomarker	Sex*	Ref†	15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours	ICC
n	-	34	34	34	34	34	34	34	33	33	33	33	33	33	33	-
Glucose and insulin																
Glucose‡	M	5·40 mmol/L	40·4	41·6	21·5	7·00	−4·49	−6·47	−5·41	−6·00	−8·37	−13·3	−13·8	−13·3	−15·1	0·09
	F	5·12 mmol/L	43·3	40·6	18·4	10·3	5·01	−3·26	2·92	2·58	−4·30	−9·17	−7·54	−13·5	−12·6	0·45
Insulin	M	4·53 mIU/L	707	810	621	484	283	173	60·6	−25·9	−56·6	−57·3	−64·8	−62·6	−61·1	0·14
	F	4·37 mIU/L	792	846	641	523	312	173	81·8	7·4	−24·6	−45·8	−43·5	−42·2	−43·6	0·28
Amino acids																
Alanine	M	342 μmol/L	24·4	34·2	38·7	40·2	38·2	33·6	18·6	7·76	−6·26	−9·89	−11·0	−11·5	0·32	0·14
	F	321 μmol/L	27·4	38·5	44·3	44·9	44·0	36·0	24·1	10·5	−5·45	−10·8	−14·3	−10·8	3·75	0·50
Arginine	M	87·5 μmol/L	22·0	16·9	17·4	16·3	19·0	19·3	7·68	−1·46	−4·20	−1·96	−1·43	1·71	−0·43	0·67
	F	84·0 μmol/L	20·4	13·5	16·0	12·3	17·1	19·2	9·3	−0·87	−5·16	−2·94	−2·55	2·85	0·67	0·50
Asparagine	M	68·7 μmol/L	29·6	30·2	27·9	26·2	23·7	20·4	6·82	−2·39	−5·99	−4·44	−5·80	−6·93	−3·80	0·33
	F	64·0 μmol/L	33·5	31·1	30·5	29·1	29·1	23·7	12·7	2·26	−4·73	−2·44	−5·06	−4·58	−9·04	0·50
Aspartartic acid	M	16·1 μmol/L	−0·45	1·26	0·41	1·52	1·36	0·38	1·84	1·40	0·03	3·03	4·69	3·72	2·78	0·88
	F	19·6 μmol/L	−1·46	−1·99	−2·81	−2·36	−2·02	−1·38	3·32	3·82	3·13	3·76	5·20	3·34	−0·14	0·53
Glutamic acid	M	46·4 μmol/L	−5·16	6·76	4·10	6·17	−7·39	−9·9	−7·87	−12·7	−14·9	−16·6	−13·8	−12·0	−17·0	0·62
	F	39·6 μmol/L	−9·00	3·19	8·55	7·44	−3·54	−4·72	−4·84	−5·23	−13·4	−8·85	−7·19	−16·5	−13·9	0·51
Glutamine	M	551 μmol/L	5·45	6·03	5·68	5·51	8·48	10·3	7·51	5·47	5·97	7·32	5·82	2·44	1·53	0·73
	F	459 μmol/L	5·54	4·54	3·78	4·37	8·40	8·89	9·50	8·85	6·43	7·27	8·29	6·24	−0·82	0·50
Histidine	M	79·4 μmol/L	7·74	10·6	10·7	10·1	12·8	15·7	9·47	2·38	−1·18	−0·61	−0·61	0·48	7·32	0·92
	F	80·8 μmol/L	9·15	9·71	8·84	9·16	13·4	15·2	12·4	5·58	−1·02	−0·75	−0·24	1·41	4·00	0·50
Isoleucine	M	68·5 μmol/L	18·0	19·1	19·1	19·6	21·8	23·2	9·36	−4·24	−4·17	3·09	6·25	10·2	21·1	0·41
	F	60·4 μmol/L	16·6	16·0	17·2	18·2	27·7	34·1	28·7	14·1	5·70	10·5	15·2	20·0	36·7	0·50
Leucine	M	136 μmol/L	15·1	15·6	16·1	16·5	18·6	20·0	9·22	−0·97	0·94	7·75	11·0	13·4	33·4	0·40
	F	120 μmol/L	14·1	13·7	14·2	15·0	22·8	28·2	24·2	13·1	7·40	12·4	17·9	20·6	41·9	0·50
Lysine	M	162 μmol/L	12·4	13·3	14·0	14·7	18·4	21·3	13·9	3·81	−1·81	0·57	2·10	3·99	11·4	0·42
	F	151 μmol/L	11·3	10·8	9·91	10·4	17·4	20·7	16·5	6·00	−3·30	−1·44	0·51	2·35	6·42	0·50
Phenylalanine	M	70·3 μmol/L	10·7	13·1	15·2	17·1	19·1	17·8	5·16	−4·63	−5·37	1·52	3·88	4·84	4·89	0·33
	F	71·0 μmol/L	10·1	12·8	15·6	19·0	24·3	25·4	19·0	6·78	−1·88	2·43	3·71	4·67	5·16	0·50
Proline	M	203 μmol/L	27·2	33·7	38·8	42·7	44·1	46·2	31·5	18·0	5·64	−0·09	−5·08	−9·49	−18·3	0·33
	F	155 μmol/L	34·6	43·7	53·7	61·3	67·6	66·8	53·8	33·4	10·3	0·30	−6·37	−11·9	−20·5	0·49
Threonine	M	140 μmol/L	13·5	15·0	14·8	12·7	11·1	10·2	2·31	−6·53	−10·2	−11·3	−12·9	−16·0	−13·7	0·37
	F	125 μmol/L	17·4	16·6	13·1	13·9	15·6	12·7	6·50	−2·28	−10·1	−10·4	−12·3	−14·2	−15·3	0·51
Tryptophan	M	75·1 μmol/L	4·48	2·02	1·26	1·18	0·98	0·56	−6·94	−14·6	−18·4	−13·4	−7·70	−3·90	−12·0	0·47
	F	67·3 μmol/L	4·04	1·98	1·40	1·46	3·22	4·36	−0·53	−11·1	−17·9	−10·4	−9·16	−3·56	−9·71	0·50
Tyrosine	M	65·6 μmol/L	9·51	12·4	14·5	17·3	22·3	26·1	17·1	3·40	−9·60	−11·3	−13·0	−12·9	−10·6	0·39
	F	58·9 μmol/L	8·12	10·2	12·6	16·4	26·0	32·8	31·5	14·5	−8·54	−11·6	−13·6	−13·2	−6·95	0·51
Valine	M	259 μmol/L	7·07	8·61	8·96	8·80	10·5	12·7	8·46	2·52	−0·01	−0·01	0·27	1·22	12·4	0·68
	F	241 μmol/L	6·48	7·41	6·69	6·71	11·0	14·8	15·8	11·0	4·86	3·47	3·61	4·67	17·0	0·50
One-carbon metabolites																
Betaine	M	37·6 μmol/L	11·1	16·9	19·9	20·6	23·2	23·2	14·7	10·7	2·29	2·16	−2·50	−3·60	−3·12	0·82
	F	28·6 μmol/L	10·6	14·1	16·7	20·1	27·2	26·9	15·1	9·44	5·64	4·90	−0·81	−4·06	−14·4	0·59
Choline	M	9·34 μmol/L	12·7	10·3	7·70	8·03	3·27	3·84	2·91	3·33	0·92	6·74	−0·90	−1·79	−2·90	0·74
	F	9·04 μmol/L	14·8	10·9	9·03	7·12	5·46	5·69	4·80	−0·06	−1·28	3·61	−4·43	−13·4	−7·90	0·51
Cystathionine	M	0·18 μmol/L	−0·79	3·16	9·83	14·8	23·9	34·9	38·5	22·4	−6·81	−20·8	−25·5	−25·5	−22·3	0·75
	F	0·17 μmol/L	−0·46	5·46	9·49	10·4	19·8	31·3	38·2	26·4	−8·38	−26·3	−31·2	−32·9	−27·4	0·54

Table 5. (*Continued*)

Serum biomarker	Sex*	Time after meal														
		Ref†	15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours	ICC
n	-	34	34	34	34	34	34	34	33	33	33	33	33	33	33	-
Cysteine	M	257 μmol/L	−1·03	−2·80	−4·04	−4·31	−4·59	−4·62	−5·14	−3·51	0·44	5·00	7·46	9·70	11·9	0·71
	F	244 μmol/L	−0·15	−2·51	−2·84	−2·80	−3·95	−5·03	−5·38	−3·63	0·25	5·28	7·78	9·45	11·3	0·50
DMG	M	3·66 μmol/L	6·37	3·81	2·50	8·09	15·5	13·9	13·3	6·98	−2·16	−4·82	4·64	5·49	6·92	0·84
	F	3·22 μmol/L	−3·58	0·66	1·94	2·40	8·03	9·11	6·21	2·43	−5·56	−3·81	4·30	5·96	9·67	0·78
Glycine	M	260 μmol/L	7·33	7·56	6·96	6·41	8·07	7·88	2·18	−0·85	−3·18	−2·71	−2·77	−4·44	−1·40	0·67
	F	253 μmol/L	5·54	3·63	1·92	1·68	4·12	2·70	−1·79	−4·00	−6·80	−5·07	−5·02	−5·00	−5·41	0·52
tHcy	M	8·69 μmol/L	−1·62	−3·08	−4·28	−3·55	−2·76	−1·83	−0·12	0·71	2·43	6·75	8·66	10·2	13·1	0·93
	F	7·60 μmol/L	−2·37	−3·61	−3·67	−3·02	−4·21	−4·31	−1·70	−0·41	0·56	3·17	6·23	6·37	9·34	0·58
Methionine	M	30·7 μmol/L	16·3	17·9	19·5	21·4	26·3	27·3	8·66	−8·70	−14·4	−9·58	−7·81	−7·31	−5·07	0·27
	F	27·8 μmol/L	15·0	15·4	17·7	20·2	27·8	31·3	20·8	0·62	−13·5	−8·99	−8·34	−7·37	−4·24	0·50
MetSo	M	0·74 μmol/L	7·50	17·2	34·3	43·8	52·5	67·3	49·9	11·6	−0·43	−17·5	−14·3	−6·70	−19·4	0·16
	F	0·82 μmol/L	23·8	25·4	52·2	66·8	88·1	76·9	86·7	41·6	−1·30	−3·64	−21·2	−14·5	−10·5	0·23
Sarcosine	M	1·52 μmol/L	16·3	18·7	19·6	20·7	22·6	23·6	12·9	1·97	−8·51	−14·1	−17·8	−21·1	−29·1	0·58
	F	1·18 μmol/L	15·0	16·4	18·1	20·7	26·4	27·8	20·7	5·83	−9·82	−14·7	−19·3	−22·6	−31·1	0·69
Serine	M	129 μmol/L	14·0	16·2	15·5	14·3	12·2	11·7	4·13	−0·74	−2·74	−1·07	−0·42	−0·11	0·38	0·26
	F	133 μmol/L	14·3	14·8	13·4	13·3	13·4	12·5	9·25	3·28	0·24	3·62	3·51	3·16	−1·42	0·50
B-vitamin markers																
Cobalamin	M	330 pmol/L	−2·45	−3·50	−5·04	−2·33	−3·33	−5·62	−1·77	0·81	0·45	1·83	4·75	5·28	12·9	0·97
	F	325 pmol/L	−1·31	−1·08	0·11	−0·64	−1·03	−1·17	−0·86	−0·75	0·63	3·20	5·58	6·27	14·3	0·52
FMN	M	7·75 nmol/L	−23·1	−28·1	−28·3	−31·6	−29·4	−24·9	−14·0	1·42	28·1	46·7	44·2	58·9	92·1	0·43
	F	8·44 nmol/L	−14·8	−21·2	−30·0	−28·2	−23·0	−19·3	−3·70	12·2	37·0	38·4	53·9	52·3	87·4	0·54
Folate	M	14·0 nmol/L	12·6	14·3	14·7	13·6	7·89	3·68	3·94	5·22	19·9	28·6	29·0	27·8	50·2	0·63
	F	13·6 nmol/L	10·1	12·0	11·6	11·8	8·16	4·77	0·14	3·71	13·5	17·0	28·9	33·5	65·4	0·54
mNAM	M	90·6 nmol/L	17·6	16·2	10·7	7·55	13·8	16·6	6·79	12·6	20·9	20·3	3·47	−21·9	6·58	0·73
	F	126 nmol/L	24·3	19·4	9·99	10·3	6·38	−0·95	−8·31	−14·1	−9·59	−7·60	−21·7	−34·7	−4·54	0·52
MMA	M	0·13 μmol/L	0·11	−0·30	1·24	3·47	5·24	7·02	5·52	−2·56	−12·0	−14·6	−15·6	−15·9	−14·1	0·71
	F	0·14 μmol/L	2·72	0·65	0·89	0·56	2·01	4·70	2·51	−3·32	−14·0	−17·7	−21·6	−17·3	−9·33	0·61
Nicotinamide	M	194 nmol/L	52·9	45·8	32·8	13·6	5·94	−2·50	−4·82	7·25	14·3	5·74	−4·30	−30·0	−11·4	0·60
	F	214 nmol/L	23·8	3·70	−14·9	−23·7	−31·1	−34·8	−39·4	−27·5	−25·4	−23·6	−29·5	−36·6	−10·2	0·52
Pyridoxal	M	18·9 nmol/L	9·08	10·6	11·0	8·92	4·21	3·30	−5·09	−4·60	−6·69	−8·19	−6·98	−7·51	−8·71	0·87
	F	15·2 nmol/L	12·7	16·1	16·8	17·8	11·6	10·8	5·44	−2·71	−2·52	−2·42	−3·14	−2·79	−7·83	0·54
PLP	M	61·9 nmol/L	−3·04	−4·85	−7·50	−8·39	−11·2	−11·1	−12·5	−11·9	−13·1	−14·4	−16·5	−17·8	−13·0	0·95
	F	47·2 nmol/L	−3·06	−5·74	−7·75	−9·39	−9·55	−11·5	−8·85	−9·7	−11·9	−14·2	−18·3	−21·7	−17·2	0·53
4-pyridoxic acid	M	19·6 nmol/L	−4·03	−7·59	−17·4	−18·0	−22·8	−24·6	−22·9	−17·6	−16·2	−22·9	−21·6	−25·3	2·97	0·68
	F	18·2 nmol/L	−2·01	−9·60	−19·2	−20·9	−26·6	−29·0	−26·9	−27·8	−27·6	−27·3	−27·8	−28·2	3·77	0·52
Riboflavin	M	11·2 nmol/L	−1·23	1·50	3·10	1·99	1·17	−3·56	−5·57	−6·46	1·51	−0·71	8·52	13·4	29·4	0·94
	F	16·5 nmol/L	−0·06	0·89	2·02	4·20	−2·19	1·80	−2·94	1·28	4·87	4·92	14·7	12·6	39·4	0·64
Thiamine	M	7·01 nmol/L	21·6	24·5	21·8	20·1	15·5	9·87	2·24	1·64	−0·81	−2·75	−1·43	−7·24	−17·8	0·69
	F	7·73 nmol/L	17·3	23·0	20·3	18·3	14·1	8·01	9·38	6·09	−1·52	−1·29	−5·08	−13·8	−22·2	0·56
TMP	M	3·04 nmol/L	12·9	7·66	5·97	1·26	2·26	2·09	11·2	19·3	17·5	12·8	0·61	5·92	−7·79	0·79
	F	3·05 nmol/L	9·65	1·65	3·19	−3·30	−10·3	−11·0	4·15	9·35	9·65	−6·52	−7·26	−19·1	−17·9	0·92

ICC, Intraclass correlation coefficient; DMG, Dimethylglycine; FMN, Flavin mononucleotide; MetSo, Methionine sulfoxide; mNAM, N^1-methyl nicotinamide; MMA, Methylmalonic acid; PLP, Pyridoxal-5´-phosphate; tHcy, Total homocysteine; TMP, Thiamine monophosphate.
*Male/Female.
† Reference values are reported as geometric means. The reference values were measured around 8 a.m., 12 hours after a standard evening meal.
‡ Capillary glucose.

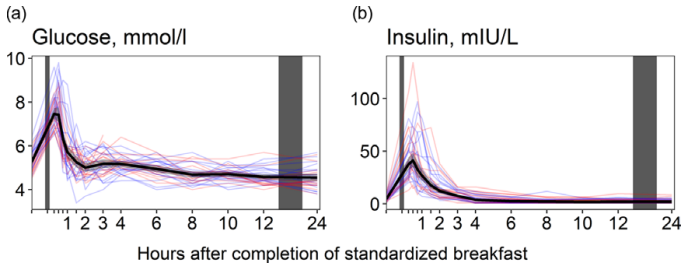

Figure 3. The concentrations of glucose and insulin as a function of time since completion of the standardised breakfast meal in participants in the Postprandial Metabolism Study ($n = 34$). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The blue and red lines represent the male and female participants, respectively. The leftmost vertical line indicates the time of the standardised breakfast meal, while the rightmost vertical line indicates time spent outside the study centre.

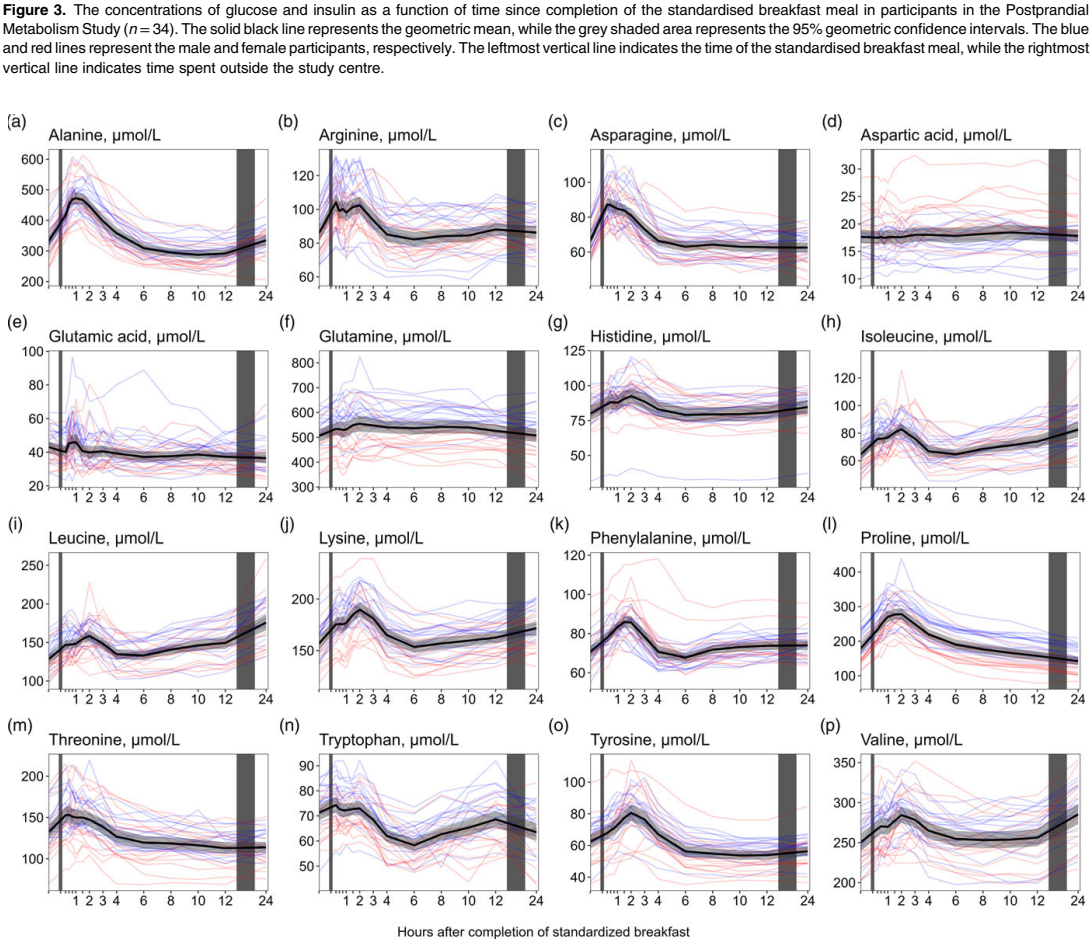

Figure 4. The concentrations of amino acids as a function of time since completion of the standardised breakfast meal in participants in the Postprandial Metabolism Study ($n = 34$). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The blue and red lines represent the male and female participants, respectively. The leftmost vertical line indicates the time of the standardised breakfast meal, while the rightmost vertical line indicates time spent outside the study centre.

betaine, choline, dimethylglycine and the amino acids cystathionine, glycine, methionine, sarcosine and serine increased and peaked within the first 3 h after completion of the meal (Fig. 5). The largest relative increase was observed for cystathionine (Fig. 5(c), +38·3 % increase from baseline values), methionine (Fig. 5(h), +29·2 %) and betaine (Fig. 5(a), +25·1 %). The concentrations were thereafter relatively stable until 24 h after the meal, except for cystathionine, which decreased to its

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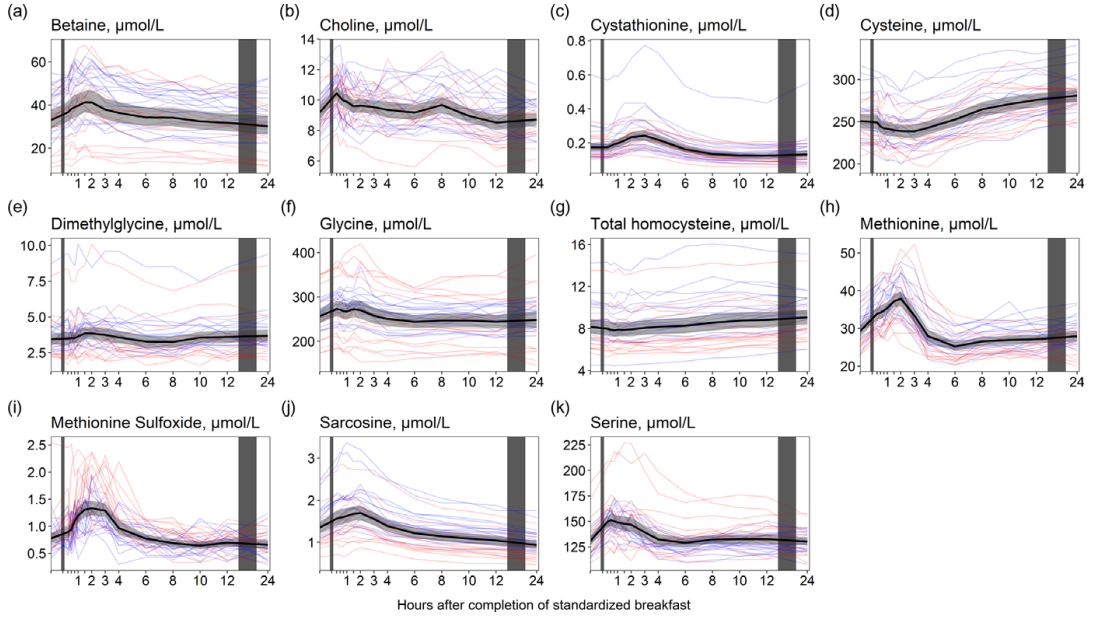

Figure 5. The concentrations of one-carbon metabolites as a function of time since completion of the standardised breakfast meal in participants in the Postprandial Metabolism Study ($n = 34$). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The blue and red lines represent the male and female participants, respectively. The leftmost vertical line indicates the time of the standardised breakfast meal, while the rightmost vertical line indicates time spent outside the study centre.

lowest values at 12 h (–29·0% decrease from baseline values). For cysteine and homocysteine (Fig. 5(d) and (g)), the opposite was observed, with slightly decreased concentrations immediately after the meal followed by increased concentrations peaking at 24 h (+11·6% and 11·4% increase, respectively). The ICC for the one-carbon metabolites ranged from 0·25 (methionine sulfoxide) to 0·82 (dimethylglycine). As with the amino acids, the males had on average slightly higher concentrations of all one-carbon metabolites except for methionine sulfoxide and serine in which the females had slightly higher concentrations. Further, we observed that the females had a higher peak in concentrations of methionine sulfoxide than males (+88·1 % at 90 minutes after the meal for females; +67·3 % at 2 h for males). The relative changes for the other one-carbon metabolites were comparable between the sexes (Table 5).

B-vitamin biomarkers

For cobalamin and folate (Fig. 6(a) and (c)), we observed no considerable changes in concentrations immediately after the meal, except for a small peak in folate concentrations in the first hour after the meal (+13·0% increase). However, the concentrations of both cobalamin and folate steadily increased from 3 h, peaking at 24 h after the meal (+13·5 % increase for cobalamin and +56·8 % increase for folate). A similar pattern was observed for FMN and riboflavin (Fig. 6(b) and (j)), with the highest concentrations at 24 h (+89·9 % and +33·8 % increase for FMN and riboflavin, respectively). For the vitamin B_6 vitamers (Fig. 6(g)–(i)), a slight increase in pyridoxal concentrations was observed right after the meal, peaking at 45 min (+13·7 % increase), with concentrations returning to baseline at 3 h and thereafter slightly decreasing until 24 h. Pyridoxal-5-phosphate concentrations decreased in the hours after the meal, with the lowest values observed at 12 h (–19·6 % decrease). The concentration of 4´-pyridoxic acid decreased right after the meal with the lowest values observed at 2 h (–26·6 % decrease) and remained decreased until 12 h but thereafter increased and returned to baseline values at 24 h. The concentrations of N1´-methylnicotinamide, thiamine and TMP (Fig. 6(d), (k), (l)) increased right after the meal, peaking within the first hour (N1´-methylnicotinamide: +20·7 % increase, thiamine: +23·8% increase, TMP: +11·4 % increase). The concentrations thereafter decreased, with N1´-methylnicotinamide reaching the lowest values at 12 h (–28·0 % decrease from baseline values) and thiamine and TMP reaching the lowest values at 24 h (thiamine: –19·8 % decrease, TMP: –12·5 %). The concentrations of methylmalonic acid (Fig. 6(e)) slightly increased the first 3 h after the meal but thereafter decreased, reaching the lowest levels at 10 h (–18·4 % decrease from baseline values). The ICC for the B-vitamin biomarkers ranged from 0·53 (FMN) to 0·88 (TMP). The results were largely similar between the sexes, except for the change in concentrations of N1´-methylnicotinamide and nicotinamide (Table 5). For N1´-methylnicotinamide, we observed that concentrations immediately increased at 15 min in females but thereafter steadily

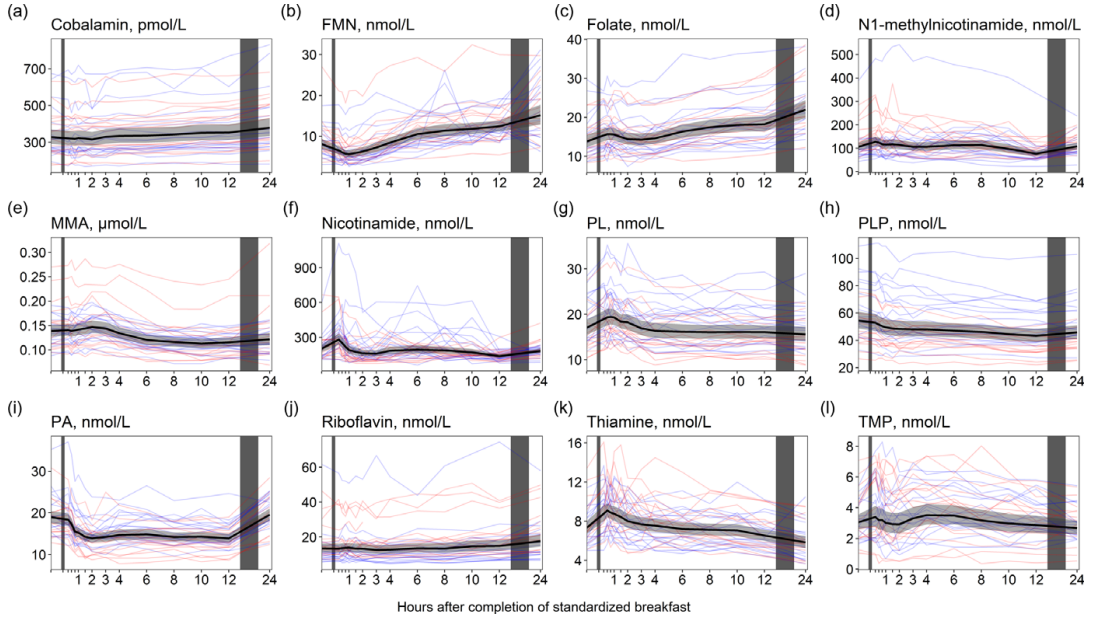

Figure 6. The concentrations of B-vitamin biomarkers as a function of time since completion of the standardised breakfast meal in participants in the Postprandial Metabolism Study ($n = 34$). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The blue and red lines represent the male and female participants, respectively. The leftmost vertical line indicates the time of the standardised breakfast meal, while the rightmost vertical line indicates time spent outside the study centre.

decreased until 12 h after the meal. In males, the concentrations increased and remained elevated until 8 h after the meal before decreasing. For nicotinamide, the concentrations slightly increased the first hour after the meal in females, and thereafter decreased and remained lowered until 12 h after the meal. In males, the concentrations increased and remained elevated or at baseline levels the first 8 h after the meal before thereafter decreasing.

Discussion

In this study of thirty-four healthy participants, we observed changes in circulating concentrations of a number of serum biomarkers and metabolites after the consumption of a standardised breakfast meal followed by food abstinence for 24 h.

Potential mechanisms and comparison with other studies

Amino acids. Consistent with previous studies, we observed that males generally exhibited higher concentrations of most serum amino acids compared with females[23,24]. However, the relative changes in amino acids were comparable between sexes. Further, our findings on amino acids are consistent with several previous studies. In a review by LaBarre and colleagues[25], they reported that the blood concentrations of amino acids tended to peak at 60–90 min after a mixed macronutrient challenge and return to baseline values 4 h after the meal. However, while most of the studies included in the LaBarre review examined the effect of specific foods or comparisons like cod *v.* beef, or rye bread *v.* wheat bread, our study demonstrates that amino acid concentrations follow this pattern even after a regular breakfast meal. The amino acid profile probably reflects the digestion and absorption of proteins, wherein proteins are cleaved into dipeptides, tripeptides and amino acids[26]. Yet, the meal amino acid composition might not fully predict the postprandial blood response. Similar to the present study, Badoud *et al.*[27] reported the largest relative increase in concentrations postprandially for proline and alanine after a high-energy breakfast. Intriguingly, the most abundant amino acids in the high-energy breakfast were leucine, glutamic acid and proline, indicating that the amino acid postprandial response cannot entirely be explained by the abundance of the amino acids eaten. Further, glutamic acid is one of the most abundant amino acids found in dietary protein[28], but in the present study, we observed only small changes in glutamic acid after the meal compared with most other amino acids, which has also been observed by others[29]. It has been suggested that glutamic acid is metabolised to various amino acids in the enterocyte, mainly alanine but also proline[28], which may explain the observations in the present study. Similarly to our observations at 24 h, Krüg *et al.*[5] and Rubio-Aliaga *et al.*[4] reported increased concentrations of the BCAA during 36 h of fasting. The BCAA cannot be synthesised *de novo* and must be obtained from the diet or by proteolysis. During fasting, the main source of BCAA in the blood is protein degradation[30], predominantly derived from skeletal

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muscle[31]. It has been suggested that increased serum concentrations of BCAA during fasting are related to decreased glycolysis and increased fatty acid oxidation and proteolysis in muscles[32].

One-carbon metabolites. Among the one-carbon metabolites, serum concentrations of cystathionine and methionine were most responsive to food intake and subsequent fasting. It is well known that methionine levels increase and peak 1 h after a methionine loading test[33]. Although the methionine content of the breakfast meal in the present study was lower than that typically ingested in a methionine loading test, the breakfast meal (including cheese and whole grains) likely caused the observed increase in methionine, which peaked at 2 h after the meal. Methionine can be converted to homocysteine through the methionine–homocysteine cycle, which may further be converted to cystathionine in the transsulphuration pathway[34]. It has previously been shown that cystathionine concentrations increase after the intake of methionine[35]. In the present study, the temporal pattern is consistent with methionine being released from protein in the food, followed by an increase in cystathionine, peaking at 3 h after the meal. We also observed increased concentrations of betaine, choline, dimethylglycine, glycine and sarcosine in the first hours after the meal. All these metabolites are involved in the choline oxidation pathway. Calculations using data from the USDA food database[36] suggest that the betaine and choline content in the breakfast was approximately 113 and 25 mg, respectively, which may explain the increased concentrations of the metabolites in the choline oxidation pathway. Interestingly, we observed slightly decreased concentrations of homocysteine and cysteine after the meal, contrary to the findings for the other one-carbon metabolites. This has been reported previously at 1 and 2 h after a meal[18] and may have several possible explanations. First, increased availability of choline and betaine may facilitate the remethylation of homocysteine to methionine using betaine or 5-methyltetrahydrofolate as a methyl donor. Second, the decreased concentrations of homocysteine and cysteine, accompanied by increased concentrations of cystathionine immediately after the meal, may indicate an increased conversion of homocysteine to cystathionine through the transsulfuration pathway.

B-vitamin biomarkers. In this study, we observed a sharp increase in thiamine and TMP concentrations immediately after the meal, consistent with our previous findings using cross-sectional data[37]. Both free thiamine and TMP enter the bloodstream during the absorption of thiamine[38], and the observed peak might be attributable to the thiamine content from the meal, which is estimated to be about 0·54 mg (Table 2). Further, we observed a sharp decrease in FMN concentrations, reaching the lowest value (–30·0 % decrease) at 1 h after the meal, similar to what has been reported previously[18,37,39]. FMN serves as a cofactor in the electron transport chain, and the sharp decrease may indicate increased utilisation as a cofactor in the first hour after a meal. We observed increases in FMN and riboflavin concentrations from 4 to 24 h. To our knowledge, no studies have previously reported changes in FMN or riboflavin concentrations in the fasting state. As the increase in both FMN and riboflavin concentrations started around 4 h after the meal, it is unlikely the increase was due to the riboflavin content of the meal. We observed a similar pattern for folate, which was 56·8 % higher at 24 h compared with baseline values. Similar observations have been reported previously, with a doubling in folate concentrations following 36 h of fasting compared with immediately after a meal. It has been suggested that this increase may be explained by reduced excretion of folate in bile during fasting[40].

Implications

We have demonstrated that several metabolites and biomarkers change dynamically after a habitual meal in healthy, young adults. This could have implications in the clinic, where specific cut-offs in circulating biomarker concentrations are used to diagnose a disease or condition or to monitor or initiate treatment. For example, we observed that folate concentrations increased on average by 56·8 % from baseline values to 24 h. A total of six participants (17 %) had folate concentrations below the established cut-off at 10 nmol/l set by the WHO[41] at baseline or during the first hours after the meal. However, at later timepoints, all these subjects had folate concentrations above this cut-off, meaning in a clinical setting they would have been classified as folate deficient if their blood sample was taken before or during the first hours after the meal. Our findings indicate that clinically it is important to accurately account for prandial status and time since last meal when evaluating certain serum biomarkers. This can be done by standardising the blood sampling timepoint, by utilising data on the dynamics of postprandial metabolism or by applying different cut-offs according to the time since food intake. Further, our findings could have implications for observational research where the time of blood sampling is rarely standardised to account for dietary intake and time since last meal. Not standardising the time of blood sampling but only distinguishing between 'fasting' and 'non-fasting' states when investigating metabolite concentrations in research may introduce measurement error. When the metabolite concentration is modelled as the independent variable, non-differential measurement error is, on average, expected to attenuate the observed associations due to regression dilution bias[42]. However, attenuation cannot be automatically assumed in individual studies, as the measurement error may be unequally distributed by chance[43], or if the concentration is grouped into categories, such as quantile groups[42]. Further, if the biomarker is modelled as a confounder, non-differential measurement error may result in residual confounding, biasing the association in the same direction as the original confounding[42]. In this context, non-differential measurement error may result from blood samples collected at random timepoints after food intake. Differential measurement error of the biomarker, potentially arising from systematically collecting the blood samples at timepoints associated with peak or through metabolite concentration, could bias the risk association in any direction and give rise to wrong conclusions from the study[10]. We suggest that in future epidemiological studies, blood sampling in relation to time since last meal should be standardised to limit the impact of prandial status on circulating biomarker concentrations. When the biomarker concentration is the outcome of interest, it is crucial that sampling procedures are

comparable across participants and across timepoints when repeated measurements are taken within the same individual.

Strengths and limitations

This study has several strengths. First, we obtained data on a wide range of metabolites from thirty-four participants, including both males and females. Similar studies have usually had fewer participants[4,6] or only included male subjects[5]. In addition, to increase the internal validity of the study, we recruited a homogenous group of participants, reducing potential variability in metabolite concentrations linked to age, health status and body composition and all participants remained inactive during the study visit. Unfortunately, this reduces the generalisability of our findings. Results for glucose and insulin were highly consistent with the *a priori* expectations[4,5] and can be used as a compliance measure. Despite the homogeneity of the cohort, there were large inter-individual differences in RMR (ranging from 1053 to 2200 kcal/d) and body composition (fat mass percentage ranging from 10·5 to 41·2) between the participants. It is well known that body composition may affect postprandial responses[44]; thus, it is likely that the meal was metabolised at different rates, which introduces a source of variability in the results.

Conclusion

We observed that the circulating concentration of several metabolites changed considerably after the consumption of a standardised breakfast meal. The changes were not limited only to the hours immediately after the meal, with several metabolites changing considerably during fasting for 24 h. Our findings challenge the current, imprecise, practice of distinguishing between fasting and non-fasting blood samples and have implications for using metabolites in clinical practice and in research.

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The author's contributions were as follows: Å. M. A., H. R. R., O. K. N., J. D. and V. L. designed the study; Å. M. A., C. O. J., V. H. M. and V. L. conducted the study; A. M. C. analysed the blood samples; Å. M. A. and V. L. analysed the data and Å. M. A. wrote the paper and had primary responsibility for the final content. All authors have read and approved the final manuscript.

There are no conflicts of interest.

Data described in the manuscript, code book and analytic code will be made publicly and freely available without restriction at dataverse.uib.no.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114523002490

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Supplementary Table 1. An overview of all metabolites, analytical method, and the minimum, maximum, and mean baseline concentrations

	Laboratory (platform)[1]	Method	gMean (gSD)	Mean (SD)	Minimum	Maximum
Amino acids						
Serum alanine, µmol/L	Bevital (B)	GC-MS/MS	331 (1.18)	336 (57.5)	243	484
Serum arginine, µmol/L	Bevital (C)	LC-MS/MS	85.8 (1.15)	86.6 (12.0)	66.8	107
Serum asparagine, µmol/L	Bevital (B)	GC-MS/MS	66.5 (1.14)	67.0 (8.77)	53.7	84.9
Serum aspartate, µmol/L	Bevital (B)	GC-MS/MS	17.7 (1.22)	18.0 (3.54)	9.96	28.5
Serum glutamate, µmol/L	Bevital (B)	GC-MS/MS	43.1 (1.25)	44.1 (9.44)	26.9	63.6
Serum glutamine, µmol/L	Bevital (B)	GC-MS/MS	506 (1.16)	511 (70.9)	350	632
Serum histidine, µmol/L	Bevital (B)	GC-MS/MS	80.1 (1.21)	81.3 (12.0)	31.5	101
Serum isoleucine, µmol/L	Bevital (B)	GC-MS/MS	63.6 (1.20)	65.6 (11.9)	45.1	90.1
Serum leucine, µmol/L	Bevital (B)	GC-MS/MS	128 (1.16)	129 (18.8)	97.1	167
Serum lysine, µmol/L	Bevital (B)	GC-MS/MS	157 (1.13)	158 (18.6)	118	203
Serum phenylalanine, µmol/L	Bevital (B)	GC-MS/MS	70.6 (1.12)	71.1 (8.29)	54.7	99.0
Serum proline, µmol/L	Bevital (B)	GC-MS/MS	179 (1.27)	184 (43.3)	112	290
Serum threonine, µmol/L	Bevital (B)	GC-MS/MS	132 (1.23)	135 (27.1)	69.8	189
Serum tryptophan, µmol/L	Bevital (B)	GC-MS/MS	71.3 (1.14)	71.9 (8.83)	47.4	85.3
Serum tyrosine, µmol/L	Bevital (B)	GC-MS/MS	62.4 (1.23)	63.6 (12.3)	35.7	99.2
Serum valine, µmol/L	Bevital (B)	GC-MS/MS	250 (1.14)	252 (32.6)	202	325
One-carbon metabolites						
Serum betaine, µmol/L	Bevital (C)	LC-MS/MS	33.0 (1.49)	35.3 (11.6)	11.3	54.7
Serum choline, µmol/L	Bevital (C)	LC-MS/MS	9.19 (1.16)	9.29 (1.38)	6.35	12.9
Serum cystathionine, µmol/L	Bevital (B)	GC-MS/MS	0.18 (1.46)	0.19 (0.09)	0.11	0.60
Serum cysteine, µmol/L	Bevital (B)	GC-MS/MS	251 (1.11)	252 (25.8)	209	316

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	Laboratory (platform)[1]	Method	gMean (gSD)	Mean (SD)	Minimum	Maximum
Serum DMG, µmol/L	Bevital (C)	LC-MS/MS	3.45 (1.38)	3.65 (1.42)	2.23	8.72
Serum glycine, µmol/L	Bevital (B)	GC-MS/MS	257 (1.20)	261 (47.4)	173	352
Serum total homocysteine, µmol/L	Bevital (B)	GC-MS/MS	8.16 (1.28)	8.40 (2.12)	4.53	14.3
Serum methionine, µmol/L	Bevital (B)	GC-MS/MS	29.3 (1.16)	29.6 (4.44)	22.8	38.9
Serum methionine sulfoxide, µmol/L	Bevital (C)	LC-MS/MS	0.78 (1.40)	0.83 (0.37)	0.14	2.54
Serum sarcosine, µmol/L	Bevital (B)	GC-MS/MS	1.35 (1.36)	1.41 (0.44)	0.65	2.48
Serum serine, µmol/L	Bevital (B)	GC-MS/MS	131 (1.12)	132 (15.8)	110	188
B-vitamin markers						
Serum cobalamin, pmol/L	MBF	Immunoassay	328 (1.43)	350 (134)	176	675
Serum FMN, nmol/L	Bevital (D)	LC-MS/MS	8.07 (1.56)	8.91 (4.51)	3.07	27.1
Serum folate, nmol/L	MBF	Immunoassay	13.8 (1.40)	14.6 (5.30)	8.40	29.7
Serum N1-methylnicotinamide, nmol/L	Bevital (D)	LC-MS/MS	106 (1.60)	120 (70.4)	44.6	388
Serum MMA, µmol/L	Bevital (B)	GC-MS/MS	0.14 (1.30)	0.14 (0.04)	0.09	0.27
Serum nicotinamide, nmol/L	Bevital (D)	LC-MS/MS	203 (1.59)	229 (133)	109	668
Serum pyridoxal, nmol/L	Bevital (D)	LC-MS/MS	17.1 (1.33)	17.8 (5.19)	10.3	28.4
Serum PLP, nmol/L	Bevital (D)	LC-MS/MS	54.5 (1.39)	57.4 (19.3)	26.6	109
Serum 4′-pyridoxic acid, nmol/L	Bevital (D)	LC-MS/MS	18.9 (1.26)	19.5 (4.86)	12.8	35.2
Serum riboflavin, nmol/L	Bevital (D)	LC-MS/MS	13.5 (1.76)	16.1 (12.0)	5.54	61.7
Serum thiamine, nmol/L	Bevital (D)	LC-MS/MS	7.34 (1.30)	7.59 (2.10)	5.00	13.4
Serum TMP, nmol/L	Bevital (D)	LC-MS/MS	3.04 (1.58)	3.36 (1.46)	1.05	7.07
Routine clinical markers						
Serum albumin, g/L	MBF	Photometry	45.3 (1.06)	45.4 (2.45)	40.0	49.0

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	Laboratory (platform)[1]	**Method**	**gMean (gSD)**	**Mean (SD)**	**Minimum**	**Maximum**
Serum ALAT, U/L	MBF	Photometry	21.3 (1.43)	22.8 (10.7)	10.0	73.0
Serum ASAT, U/L	MBF	Photometry	24.0 (1.43)	25.9 (12.7)	16.0	74.0
Serum creatine, µmol/L	Bevital (C)	LC-MS/MS	19.2 (1.38)	20.2 (7.18)	10.2	42.2
Serum creatinine, µmol/L	MBF	Photometry	74.8 (1.19)	76.0 (13.8)	52.0	112
Serum creatinine, µmol/L	Bevital (C)	LC-MS/MS	78.9 (1.18)	80.1 (14.1)	58.8	120
Serum CRP, mg/L	MBF	Immuno-turbidimetry	0.86 (2.57)	1.41 (1.85)	0.16	9.00
Erythrocytes, 10^{12}/L	MBF	Flow cytometry	4.57 (1.09)	4.59 (0.40)	3.90	5.40
Serum gamma-glutamyltransferase, U/L	MBF	Photometry	16.0 (1.53)	17.9 (10.9)	9.00	65.0
Capillary glucose, mmol/L	-	Fingerprick, HemoCue 201 RT	5.27 (1.10)	5.29 (0.49)	4.50	6.40
Hemoglobin, g/dL	MBF	Photometry	13.8 (1.09)	13.8 (1.21)	11.8	17.3
HbA1c, mmol/mol	MBF	Immuno-agglutination	31.3 (1.11)	31.4 (3.10)	24.0	39.0
Serum insulin, mIU/L	MBF	Immunoassay	4.45 (1.94)	5.34 (3.0)	1.0	11.2
Mean corpuscular hemoglobin, pg	MBF	Calculated	30.1 (1.05)	30.2 (1.54)	26.0	34.0
Mean corpuscular volume, fL	MBF	Flow cytometry	89.8 (1.04)	89.9 (3.82)	81.0	100
Mean platelet volume, fL	MBF	Flow cytometry	10.4 (1.09)	10.5 (0.88)	9.00	12.7
Thrombocytes, 10^{9}/L	MBF	Impedance and flow cytometry	230 (1.22)	234 (45.4)	153	354
Serum TSH, mIU/L	MBF	Immunoassay	2.39 (1.58)	2.63 (1.09)	0.79	5.06
Serum 25-hydroxyvitamin D, nmol/L	MBF	Immunoassay	65.4 (1.33)	67.9 (18.9)	38.0	109
Serum HDL cholesterol, mmol/L	MBF	Photometry	1.55 (1.24)	1.58 (0.36)	1.00	2.80

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	Laboratory (platform)[1]	Method	gMean (gSD)	Mean (SD)	Minimum	Maximum
Serum LDL cholesterol, mmol/L	MBF	Photometry	2.51 (1.31)	2.61 (0.72)	1.60	4.30
Serum triglycerides, mmol/L	MBF	Photometry	0.85 (1.46)	0.92 (0.36)	0.50	1.82
Serum magnesium, mmol/L	MBF	Photometry	0.81 (1.06)	0.82 (0.04)	0.73	0.91
Serum phosphate, mmol/L	MBF	Photometry	1.14 (1.13)	1.14 (0.13)	0.75	1.39
Serum potassium, mmol/L	MBF	Indirect ion-selective electrode	4.07 (1.07)	4.08 (0.28)	3.30	4.70

Abbreviations: gMean, geometric mean; gSD, geometric standard deviation; GC-MS/MS, Gas chromatography mass spectrometry; LC-MS/MS, Liquid chromatography mass spectrometry; DMG, Dimethylglycine; MBF, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital; FMN, Flavin mononucleotide; MMA, Methylmalonic acid; PLP, Pyridoxal-5´-phosphate; TMP, Thiamine monophosphate; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CRP, C-reactive protein; HbA1c, Glycated hemoglobin; TSH, Thyroid stimulating hormone; LDL, Low-density lipoprotein; HDL, High-density lipoprotein. [1]More information can be found at https://bevital.no/ (Bevital) and at https://helse-bergen.no/en/avdelinger/laboratorieklinikken/medisinsk-biokjemi-og-farmakologi (MBF).

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Supplementary Table 2. The metabolite concentrations after the consumption of a standardized meal in healthy subjects in the Postprandial Metabolism Study[1]

Serum biomarker	Time after meal													
	Ref	**15 min**	**30 min**	**45 min**	**60 min**	**90 min**	**2 hours**	**3 hours**	**4 hours**	**6 hours**	**8 hours**	**10 hours**	**12 hours**	**24 hours**
n	34	34	34	34	34	34	34	33	33	33	33	33	33	33
Glucose and insulin														
Glucose, mmol/L[2]	5.27	7.47	7.42	6.32	5.72	5.26	5.00	5.18	5.18	4.95	4.69	4.71	4.58	4.55
Insulin, mIU/L	4.45	37.7	41.3	32.5	26.8	17.6	12.1	7.38	3.81	2.42	2.07	1.89	1.98	2.00
Amino acids														
Alanine, µmol/L	332	417	452	469	472	468	447	397	358	309	295	287	292	334
Arginine, µmol/L	85.8	104	99.0	100	98.2	101	102	93.4	85.2	82.2	84.1	84.5	88.1	86.2
Asparagine, µmol/L	66.5	87.3	86.8	85.8	84.8	83.8	81.0	73.0	66.5	63.1	64.3	63.0	62.8	62.5
Aspartate, µmol/L	17.7	17.5	17.6	17.5	17.6	17.6	17.6	18.0	18.0	17.8	18.2	18.4	18.2	17.8
Glutamate, µmol/L	43.1	40.1	45.3	45.7	46.0	40.7	39.9	40.5	39.3	37.2	37.6	38.6	37.2	36.6
Glutamine, µmol/L	506	534	533	530	531	549	555	548	540	536	542	540	526	507
Histidine, µmol/L	80.1	86.8	88.2	87.9	87.8	90.5	92.5	88.6	83.0	79.0	79.4	79.6	80.6	84.6
Isoleucine, µmol/L	64.6	75.8	76.0	76.4	76.8	80.5	82.8	76.0	66.9	64.7	68.7	71.2	73.9	82.6
Leucine, µmol/L	128	147	147	148	148	154	159	148	135	133	141	146	149	176
Lysine, µmol/L	157	175	176	176	177	185	190	181	165	153	157	160	162	172
Phenylalanine, µmol/L	70.6	78.0	79.8	81.5	83.3	85.8	85.7	78.3	70.7	67.7	71.8	73.1	73.8	73.9
Proline, µmol/L	179	234	248	261	270	277	278	249	220	190	176	166	158	142
Sarcosine, µmol/L	1.35	1.56	1.59	1.60	1.63	1.68	1.69	1.55	1.38	1.21	1.14	1.09	1.04	0.93
Threonine, µmol/L	132	153	153	151	150	150	147	139	127	120	119	116	113	114
Tryptophan, µmol/L	71.3	74.3	72.7	72.2	72.2	72.7	73.0	68.4	62.0	58.3	62.7	65.3	68.6	63.5

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Serum biomarker	Time after meal													
	Ref	**15 min**	**30 min**	**45 min**	**60 min**	**90 min**	**2 hours**	**3 hours**	**4 hours**	**6 hours**	**8 hours**	**10 hours**	**12 hours**	**24 hours**
Tyrosine, µmol/L	62.4	67.9	69.5	70.9	72.9	77.3	80.6	76.4	67.0	56.2	54.8	53.7	53.8	56.3
Valine, µmol/L	250	267	270	270	270	277	284	278	265	255	253	254	256	285
One-carbon metabolites														
Betaine, µmol/L	33.0	36.6	38.2	39.1	39.8	41.3	41.3	37.9	36.4	34.3	34.2	32.5	31.8	30.3
Choline, µmol/L	9.19	10.4	10.2	9.96	9.89	9.59	9.63	9.53	9.35	9.18	9.67	8.96	8.52	8.71
Cystathionine, µmol/L	0.18	0.17	0.18	0.19	0.20	0.21	0.23	0.24	0.22	0.16	0.13	0.13	0.12	0.13
Cysteine, µmol/L	251	249	244	242	242	240	239	239	243	253	265	271	276	281
DMG, µmol/L	3.45	3.50	3.53	3.52	3.63	3.86	3.85	3.75	3.57	3.28	3.26	3.56	3.60	3.68
Glycine, µmol/L	257	273	271	268	267	273	271	258	251	244	247	247	245	248
tHcy, µmol/L	8.16	8.00	7.89	7.83	7.89	7.88	7.91	8.07	8.16	8.27	8.56	8.75	8.83	9.06
Methionine, µmol/L	29.3	33.9	34.2	34.8	35.4	37.2	37.9	33.4	28.0	25.2	26.6	26.9	27.2	27.9
MetSo, µmol/L	0.78	0.89	0.94	1.11	1.20	1.31	1.33	1.29	0.97	0.77	0.69	0.64	0.70	0.66
Serine, µmol/L	131	150	151	150	149	148	147	139	132	129	132	133	133	130
B-vitamin markers														
Cobalamin, pmol/L	328	322	320	319	323	321	316	330	335	336	342	351	353	379
FMN, nmol/L	8.07	6.51	6.06	5.72	5.64	5.93	6.27	7.22	8.46	10.5	11.4	11.8	12.4	15.1
Folate, nmol/L	13.8	15.4	15.7	15.7	15.6	14.9	14.4	14.3	14.6	16.3	17.5	18.0	18.2	22.0
nNAM	106	128	125	117	115	117	115	106	106	113	114	97	77	108
MMA, µmol/L	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.13	0.12	0.12	0.11	0.12	0.12
Nicotinamide, nmol/L	203	281	252	219	191	176	164	160	185	194	188	172	138	184
Pyridoxal, nmol/L	17.1	18.9	19.3	19.4	19.3	18.4	18.2	16.9	16.4	16.2	16.0	16.1	16.1	15.6
PLP, nmol/L	54.5	52.8	51.6	50.3	49.6	48.8	48.3	48.1	48.0	47.1	46.2	44.6	43.4	45.9
Pyridoxic acid, nmol/L	18.9	18.4	17.3	15.5	15.3	14.3	13.9	14.2	14.7	14.8	14.2	14.3	13.9	19.6

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Serum biomarker	Time after meal													
	Ref	**15 min**	**30 min**	**45 min**	**60 min**	**90 min**	**2 hours**	**3 hours**	**4 hours**	**6 hours**	**8 hours**	**10 hours**	**12 hours**	**24 hours**
Riboflavin, nmol/L	13.5	13.4	13.6	13.8	13.9	13.4	13.3	12.6	12.8	13.6	13.4	14.7	14.9	17.6
Thiamine, nmol/L	7.34	8.77	9.09	8.88	8.75	8.43	8.00	7.68	7.55	7.21	7.14	7.06	6.54	5.84
TMP, nmol/L	3.05	3.39	3.19	3.19	3.02	2.93	2.92	3.31	3.51	3.49	3.17	2.97	2.87	2.68

Abbreviations: DMG, Dimethylglycine; FMN, Flavin mononucleotide; MetSo, Methionine sulfoxide; mNAM, N^1-methylnicotinamide; MMA, Methylmalonic acid; PLP, Pyridoxal-5´-phosphate; tHcy, Total homocysteine; TMP, Thiamine monophosphate. [1]All values are reported as geometric means [2]Capillary glucose.

Time-Resolved Metabolite Concentrations during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study. *Åslaug Matre Anfinsen*

Supplementary Table 3. The metabolite concentrations after the consumption of a standardized meal in male (n= 18) and female (n = 16) participants in the Postprandial Metabolism Study[1]

			Time after meal												
Serum biomarker	**Sex[2]**	**Ref**	**15 min**	**30 min**	**45 min**	**60 min**	**90 min**	**2 hours**	**3 hours**	**4 hours**	**6 hours**	**8 hours**	**10 hours**	**12 hours**	**24 hours**
n		34	34	34	34	34	34	34	33	33	33	33	33	33	33
Glucose and insulin															
Glucose, mmol/L[3]	**M**	5.40	7.59	7.65	6.56	5.78	5.16	5.05	5.11	5.08	4.95	4.68	4.66	4.68	4.59
	F	5.12	7.33	7.17	6.06	5.64	5.37	4.95	5.27	5.29	4.94	4.69	4.77	4.46	4.51
Insulin, mIU/L	**M**	4.53	36.5	41.2	32.7	26.4	17.3	12.3	7.27	3.36	1.97	1.93	1.59	1.69	1.76
	F	4.37	39.0	41.3	32.4	27.2	18.0	11.9	7.51	4.43	3.11	2.24	2.33	2.39	2.33
Amino acids															
Alanine, µmol/L	**M**	342	425	459	474	479	472	457	405	368	320	308	304	303	343
	F	321	409	444	463	465	462	436	388	346	296	279	268	279	325
Arginine, µmol/L	**M**	87.5	107	102	103	102	104	104	94.2	86.2	83.8	85.8	86.2	89.0	87.1
	F	84.0	101	95.4	97.5	94.4	98.4	100	92.5	83.9	80.3	82.2	82.5	87.1	85.2
Asparagine, µmol/L	**M**	68.7	89.0	89.5	87.9	86.7	85.0	82.7	73.4	67.1	64.6	65.7	64.7	64.0	66.1
	F	64.0	85.4	83.9	83.5	82.7	82.6	79.1	72.4	65.7	61.2	62.7	61.0	61.3	58.5
Aspartate, µmol/L	**M**	16.1	16.0	16.3	16.1	16.3	16.3	16.1	16.4	16.3	16.1	16.6	16.8	16.7	16.5
	F	19.6	19.3	19.2	19.1	19.2	19.2	19.3	20.2	20.3	20.2	20.3	20.6	20.2	19.5
Glutamate, µmol/L	**M**	46.4	44.0	49.5	48.3	49.3	43.0	41.8	42.8	40.5	39.5	38.7	40.0	40.9	38.5
	F	39.6	36.1	40.9	43.0	42.6	38.2	37.8	38.0	37.8	34.6	36.4	37.0	33.3	34.3
Glutamine, µmol/L	**M**	551	582	585	583	582	598	608	593	582	584	592	584	565	560
	F	459	485	480	477	479	498	500	498	495	484	488	492	483	451
Histidine, µmol/L	**M**	79.4	85.5	87.8	87.9	87.4	89.6	91.9	86.9	81.3	78.5	78.9	78.9	79.8	85.2

Time-Resolved Metabolite Concentrations during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study. *Åslaug Matre Anfinsen*

Serum biomarker	Sex[2]	Time after meal: Ref	15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours
n		34	34	34	34	34	34	34	33	33	33	33	33	33	33
	F	80.8	88.2	88.7	88.0	88.2	91.7	93.1	90.6	85.1	79.7	80.0	80.4	81.7	83.8
Isoleucine, µmol/L	**M**	68.5	80.9	81.6	81.6	81.9	83.5	84.4	74.9	65.6	65.7	70.6	72.8	75.5	83.0
	F	60.4	70.5	70.1	70.8	71.5	77.2	81.1	77.3	68.6	63.5	66.4	69.2	72.1	82.1
Leucine, µmol/L	**M**	136	156	157	158	158	161	163	148	134	137	146	151	154	181
	F	120	137	136	137	138	147	154	148	135	128	134	141	144	169
Lysine, µmol/L	**M**	162	182	183	184	186	192	196	184	168	159	163	165	168	180
	F	151	168	167	166	167	178	182	177	161	147	150	153	156	162
Phenylalanine, µmol/L	**M**	70.3	77.8	79.5	80.9	82.3	83.7	82.8	73.9	67.0	66.5	71.3	73.0	73.7	73.7
	F	71.0	78.2	80.1	82.1	84.6	88.3	89.1	84.0	75.4	69.3	72.3	73.2	73.9	74.2
Proline, µmol/L	**M**	203	258	272	282	290	293	297	267	240	215	203	193	184	166
	F	155	209	223	238	250	260	259	229	198	164	149	139	131	118
Sarcosine, µmol/L	**M**	1.52	1.77	1.81	1.82	1.84	1.87	1.88	1.72	1.55	1.39	1.31	1.25	1.20	1.08
	F	1.18	1.35	1.37	1.39	1.42	1.49	1.50	1.37	1.20	1.03	0.97	0.92	0.88	0.78
Threonine, µmol/L	**M**	140	159	161	160	157	155	154	143	131	126	124	122	117	121
	F	125	146	145	141	142	144	140	133	122	113	112	110	108	106
Tryptophan, µmol/L	**M**	75.1	78.4	76.6	76.0	76.0	75.8	75.5	69.9	64.1	61.2	65.0	69.3	72.1	66.1
	F	67.3	70.0	68.6	68.2	68.3	69.4	70.2	66.6	59.5	55.0	60.0	60.8	64.6	60.4
Tyrosine, µmol/L	**M**	65.6	71.8	73.7	75.1	77.0	80.2	82.7	76.8	67.8	59.3	58.2	57.1	57.2	58.6
	F	58.9	63.7	64.9	66.4	68.6	74.2	78.2	75.9	66.1	52.8	51.0	49.9	50.1	53.7
Valine, µmol/L	**M**	259	277	281	282	282	286	292	281	265	259	259	260	262	291
	F	241	257	259	257	257	267	276	276	264	250	246	247	249	279

Time-Resolved Metabolite Concentrations during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study. *Åslaug Matre Anfinsen*

Serum biomarker	Sex[2]	Ref	Time after meal: 15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours
n		34	34	34	34	34	34	34	33	33	33	33	33	33	33
One-carbon metabolites															
Betaine, µmol/L	**M**	37.6	41.7	43.9	45.1	45.3	46.3	46.3	43.1	41.6	38.4	38.4	36.6	36.2	36.4
	F	28.6	31.6	32.6	33.3	34.3	36.4	36.2	32.6	31.0	29.9	29.7	28.1	27.1	24.2
Choline, µmol/L	**M**	9.34	10.5	10.3	10.1	10.1	9.64	9.70	9.61	9.65	9.42	9.97	9.25	9.17	9.07
	F	9.04	10.4	10.0	9.85	9.68	9.53	9.55	9.44	9.01	8.90	9.34	8.61	7.81	8.30
Cystathionine, µmol/L	**M**	0.18	0.18	0.19	0.20	0.21	0.23	0.25	0.26	0.23	0.17	0.15	0.14	0.14	0.14
	F	0.17	0.17	0.18	0.18	0.18	0.20	0.22	0.23	0.21	0.15	0.12	0.11	0.11	0.12
Cysteine, µmol/L	**M**	257	254	250	246	246	245	245	244	248	258	270	276	282	287
	F	244	244	238	237	237	235	232	233	237	246	259	265	269	273
DMG, µmol/L	**M**	3.66	3.89	3.80	3.75	3.96	4.23	4.17	4.15	3.92	3.58	3.48	3.83	3.86	3.91
	F	3.22	3.11	3.24	3.28	3.30	3.48	3.51	3.32	3.20	2.95	3.00	3.26	3.31	3.43
Glycine, µmol/L	**M**	260	279	280	278	277	281	281	266	258	252	253	253	249	256
	F	253	267	262	258	257	263	260	248	243	235	240	240	240	239
tHcy, µmol/L	**M**	8.69	8.55	8.42	8.31	8.38	8.45	8.53	8.68	8.75	8.90	9.27	9.44	9.58	9.82
	F	7.60	7.42	7.33	7.32	7.37	7.28	7.27	7.40	7.50	7.57	7.77	8.00	8.01	8.23
Methionine, µmol/L	**M**	37.6	41.7	43.9	45.1	45.3	46.3	46.3	43.1	41.6	38.4	38.4	36.6	36.2	36.4
	F	27.8	32.0	32.1	32.7	33.4	35.5	36.5	33.5	27.9	24.0	25.2	25.4	25.7	26.5
MetSo, µmol/L	**M**	0.74	0.79	0.86	0.99	1.06	1.12	1.23	1.10	0.82	0.73	0.61	0.63	0.69	0.59
	F	0.82	1.02	1.03	1.25	1.37	1.55	1.46	1.55	1.18	0.82	0.80	0.65	0.71	0.74
Serine, µmol/L	**M**	129	147	150	149	147	145	144	134	128	126	128	129	129	130
	F	133	152	153	151	151	151	150	145	138	133	138	138	137	131

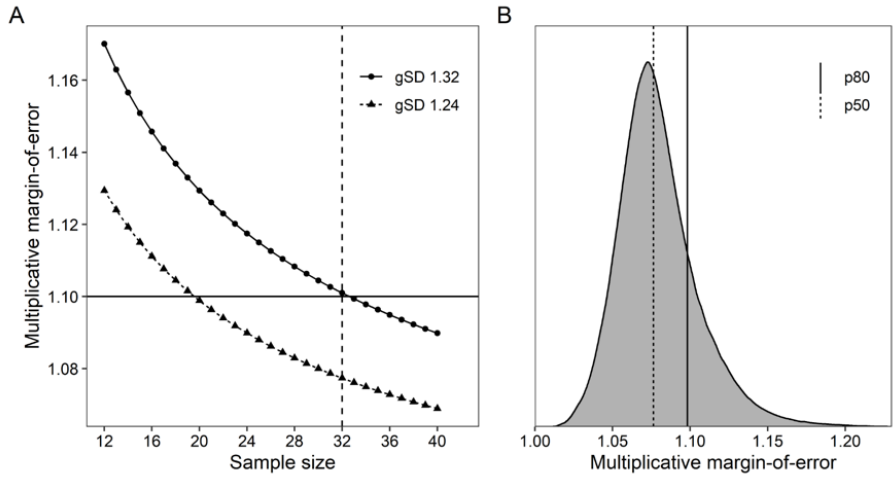
Time-Resolved Metabolite Concentrations during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study. *Åslaug Matre Anfinsen*

Serum biomarker	Sex[2]	Ref	Time after meal: 15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours
n		34	34	34	34	34	34	34	33	33	33	33	33	33	33
B-vitamin markers															
Cobalamin, pmol/L	**M**	330	322	319	314	323	319	312	325	333	332	336	346	348	373
	F	325	321	322	326	323	322	321	336	336	341	350	358	360	387
FMN, nmol/L	**M**	7.75	5.96	5.57	5.56	5.30	5.47	5.82	6.66	7.86	9.93	11.4	11.2	12.3	14.9
	F	8.44	7.19	6.65	5.91	6.06	6.50	6.82	7.94	9.24	11.3	11.4	12.7	12.5	15.4
Folate, nmol/L	**M**	14.0	15.8	16.0	16.1	15.9	15.1	14.5	14.6	14.8	16.8	18.0	18.1	17.9	21.1
	F	13.6	15.0	15.3	15.2	15.2	14.7	14.3	14.0	14.5	15.8	16.8	18.0	18.6	23.1
mNAM, nmol/L	**M**	90.6	107	105	100	97.5	103	106	97	102	110	109	93.8	70.8	96.6
	F	126	157	151	139	139	135	125	119	112	118	120	102	85	124
MMA, µmol/L	**M**	0.13	0.13	0.13	0.14	0.14	0.14	0.14	0.14	0.13	0.12	0.11	0.11	0.11	0.12
	F	0.14	0.15	0.14	0.15	0.14	0.15	0.15	0.15	0.14	0.12	0.12	0.11	0.12	0.13
Nicotinamide, nmol/L	**M**	194	296	282	257	220	205	189	184	208	221	205	185	136	171
	F	214	265	222	182	163	148	140	135	162	166	170	157	141	200
Pyridoxal, nmol/L	**M**	18.9	20.6	20.9	21.0	20.6	19.7	19.5	18.0	18.0	17.7	17.4	17.6	17.5	17.3
	F	15.2	17.1	17.6	17.8	17.9	17.0	16.9	15.8	14.5	14.6	14.6	14.5	14.5	13.8
PLP, nmol/L	**M**	61.9	60.0	58.9	57.3	56.7	54.9	55.0	54.1	54.5	53.8	53.0	51.7	50.9	53.9
	F	47.2	45.7	44.5	43.5	42.8	42.7	41.7	41.7	41.3	40.3	39.2	37.3	35.8	37.9
Pyridoxic acid, nmol/L	**M**	19.6	18.8	18.1	16.2	16.1	15.1	14.8	15.1	16.2	16.5	15.1	15.4	14.7	20.2
	F	18.2	17.8	16.5	14.7	14.4	13.4	12.9	13.2	13.1	13.1	13.2	13.1	13.0	18.8
Riboflavin, nmol/L	**M**	11.2	11.1	11.4	11.6	11.5	11.4	10.8	10.6	10.5	11.4	11.2	12.2	12.7	14.5
	F	16.5	16.5	16.6	16.8	17.2	16.1	16.8	15.5	16.1	16.7	16.7	18.3	18.0	22.2

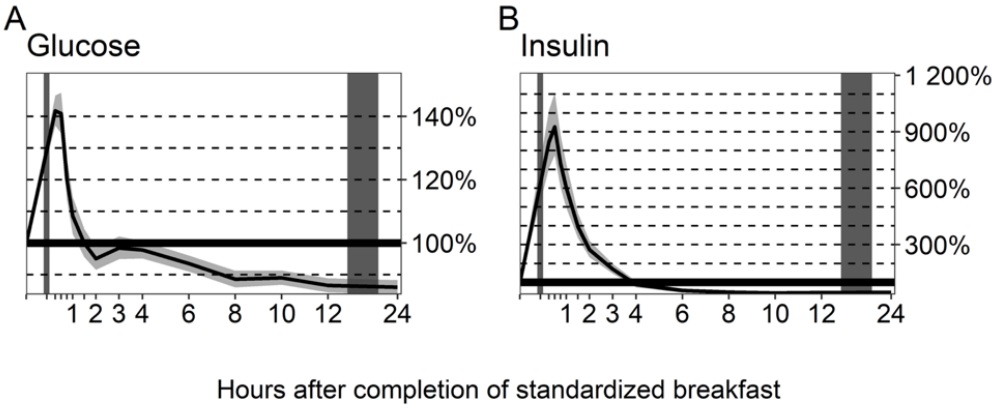
Time-Resolved Metabolite Concentrations during the Postprandial and Fasting State: The Postprandial Metabolism in Healthy Young Adults (PoMet) study. *Åslaug Matre Anfinsen*

Serum biomarker	Sex[2]	Ref	Time after meal												
			15 min	30 min	45 min	60 min	90 min	2 hours	3 hours	4 hours	6 hours	8 hours	10 hours	12 hours	24 hours
n		34	34	34	34	34	34	34	33	33	33	33	33	33	33
Thiamine, nmol/L	**M**	7.01	8.52	8.73	8.53	8.42	8.09	7.70	7.17	7.12	6.95	6.82	6.91	6.50	5.76
	F	7.73	9.06	9.50	9.29	9.14	8.82	8.34	8.36	8.10	7.52	7.54	7.25	6.59	5.94
TMP, nmol/L	**M**	3.04	3.44	3.28	3.23	3.08	3.11	3.11	3.39	3.63	3.58	3.43	3.06	3.22	2.81
	F	3.05	3.34	3.10	3.15	2.95	2.73	2.71	3.21	3.37	3.38	2.88	2.86	2.50	2.53

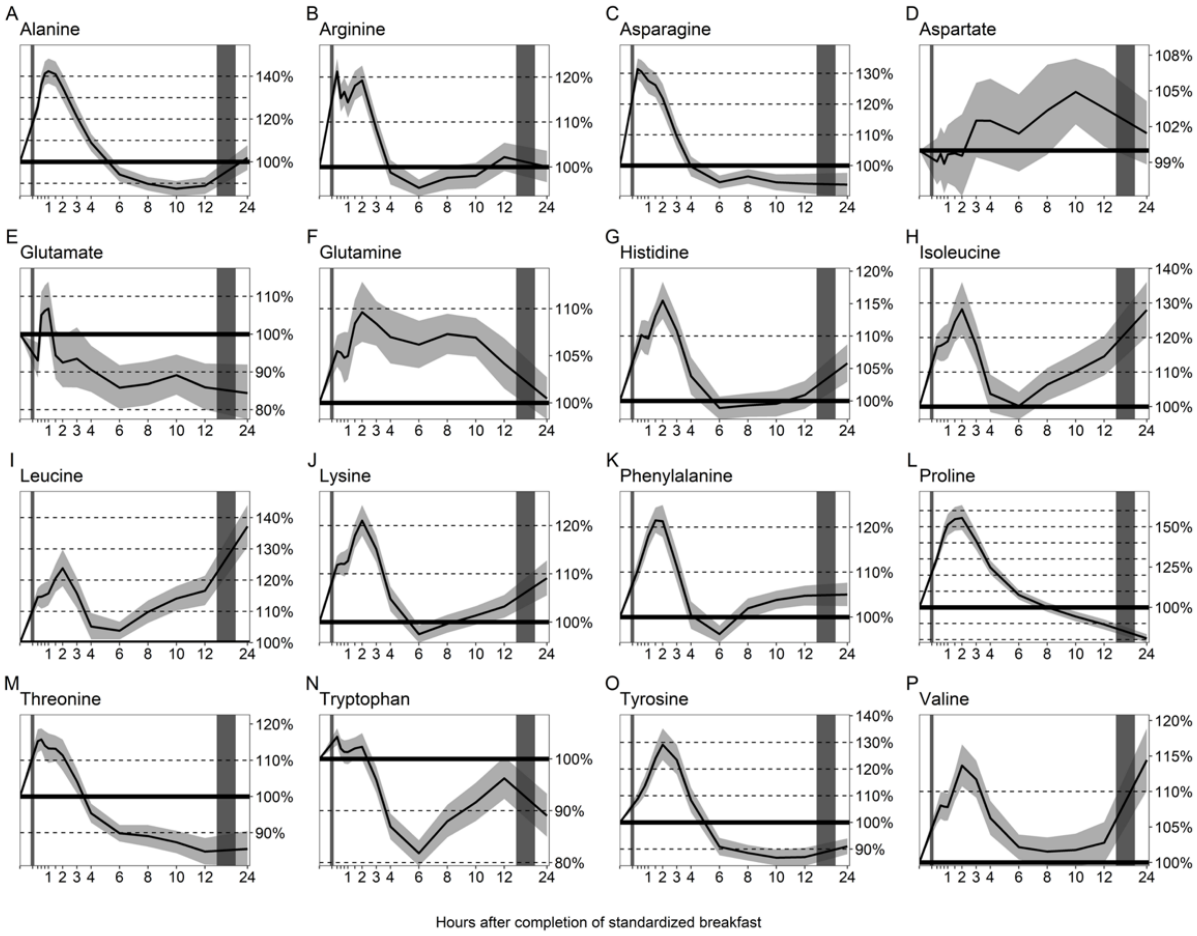
Abbreviations: DMG, Dimethylglycine; FMN, Flavin mononucleotide; MetSo, Methionine sulfoxide; mNAM, N[1]-methylnicotinamide; MMA, Methylmalonic acid; PLP, Pyridoxal-5´-phosphate; tHcy, Total homocysteine; TMP, Thiamine monophosphate. [1]All values are reported as geometric means [2]Male/Female [3]Capillary glucose.



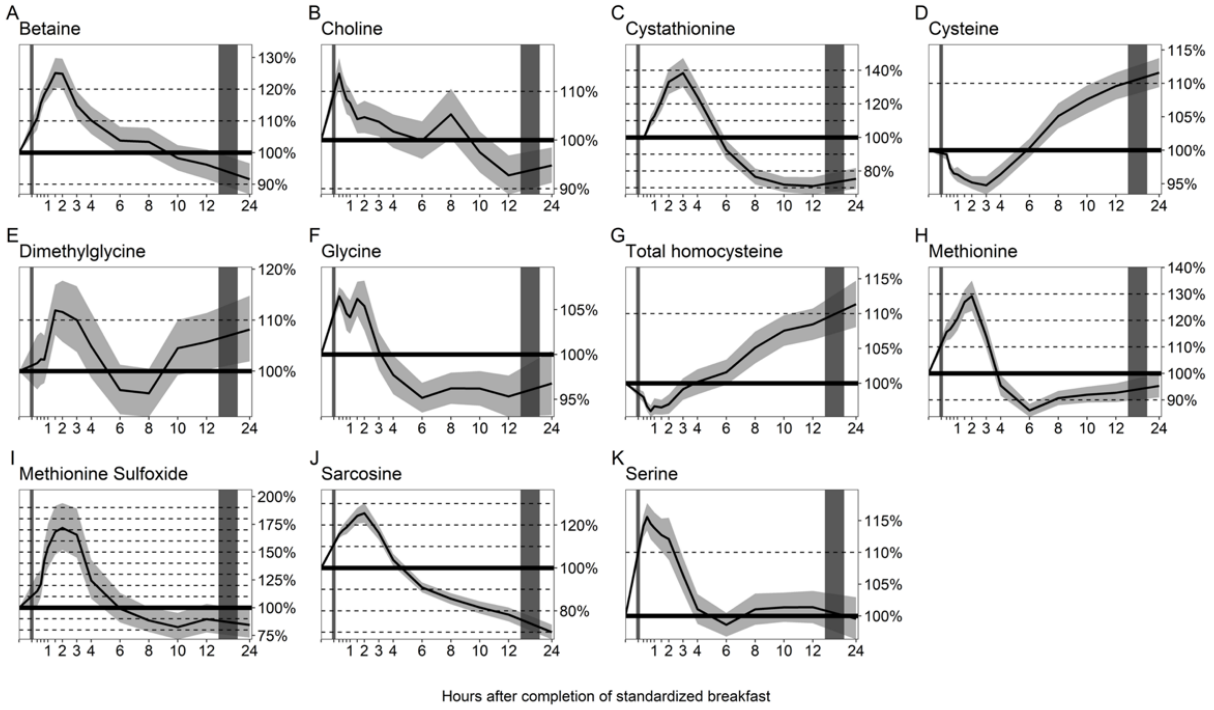
Supplementary Figure 1. A) Precision curves as a function of sample size, using the observed median (20th-80th percentile) geometric standard deviations from the HuMet study (available from http://metabolomics.helmholtz-muenchen.de/humet/). **B)** The expected distribution of multiplicative margin-of-errors for the measurements of different metabolites at different time points, with n = 32. We expected to be able to estimate the geometric mean concentrations within a multiplicative margin-of-error of 1.10 for at least 80% of all measurements. **Abbreviations:** gSD, geometric standard deviation



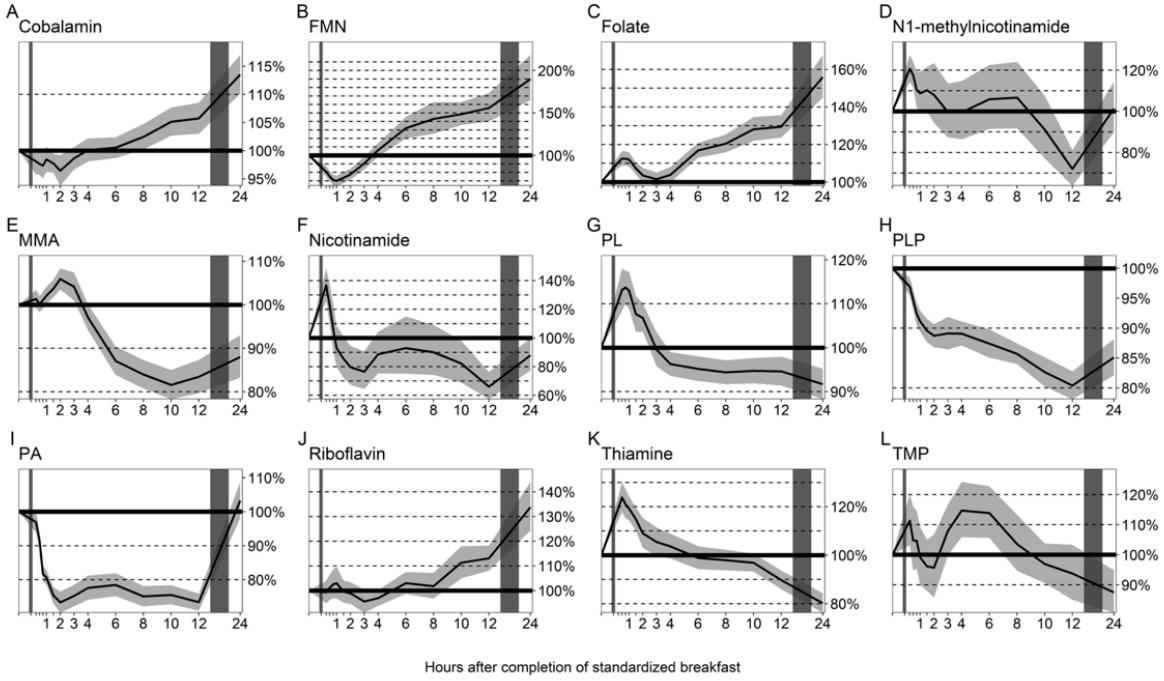
Supplementary Figure 2. The relative change in glucose- and insulin concentrations (% change from reference values) as a function of time since completion of the standardized breakfast meal in participants in the Postprandial Metabolism Study (n = 34). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The leftmost vertical line indicates the time of the standardized breakfast meal, while the rightmost vertical line indicates time spent outside the study center.



Supplementary Figure 3. The relative change in amino acid concentrations (% change from reference values) as a function of time since completion of the standardized breakfast meal in participants in the Postprandial Metabolism Study (n = 34). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The leftmost vertical line indicates the time of the standardized breakfast meal, while the rightmost vertical line indicates time spent outside the study center.



Supplementary Figure 4. The relative change in one-carbon metabolite concentrations (% change from reference values) as a function of time since completion of the standardized breakfast meal in participants in the Postprandial Metabolism Study (n = 34). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The leftmost vertical line indicates the time of the standardized breakfast meal, while the rightmost vertical line indicates time spent outside the study center.



Supplementary Figure 5. The relative change in B-vitamin biomarker concentrations (% change from reference values) as a function of time since completion of the standardized breakfast meal in participants in the Postprandial Metabolism Study (n = 34). The solid black line represents the geometric mean, while the grey shaded area represents the 95% geometric confidence intervals. The leftmost vertical line indicates the time of the standardized breakfast meal, while the rightmost vertical line indicates time spent outside the study center.
Abbreviations: FMN, Flavin mononucleotide; MMA, methylmalonic acid; PA, 4´-pyridoxic acid; PL, Pyridoxal; PLP, Pyridoxal-5´-phosphate

III

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