Lehrstuhl für Experimentelle Genetik

Targeted metabolomics analyses reveal the impact of pre-analytics and drug intake on the human metabolome

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. H. Witt
Prüfer der Dissertation:	1. apl. Prof. Dr. J. Adamski 2. Univ.-Prof. Dr. H. Daniel

Die Dissertation wurde am 16.02.2015 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 07.07.2015 angenommen.

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ABSTRACT

The pre-analytical phase of a clinical study includes all steps that are performed before the measurement of samples takes place. It is the most error-prone phase of the entire process and therefore has a major impact on the quality and validity of the test results. Little information is available about the influence of pre-analytical errors on metabolites in human study samples. Furthermore, natural fluctuations of metabolite levels complicate the interpretation of results.

In the present thesis the in vivo- and in vitro pre-analytical stability of different metabolites in serum and plasma samples of healthy humans was determined. Metabolite concentrations from six different compound classes were detected using targeted metabolomics based on liquid chromatography-tandem mass spectrometry. In the process, both reliable and stable metabolites were identified which are suitable for targeted metabolomics analysis in multicenter studies. Plasma and serum metabolites with good reliability included amino acids, biogenic amines, saturated short- and medium-chain acylcarnitines, glycerophospholipids, sphingolipids and the sum of hexoses. Except for methionine sulfoxide, serum gel-barrier tubes had no effect on the investigated metabolites, compared to serum standard tubes. In addition, it was shown that most metabolites maintained stable concentrations during simulated shipment and for up to two freeze-thaw cycles. Furthermore, a method for the evaluation of the suitability of long-term stored samples for targeted metabolomics was presented. In the investigated study cohort, long-term storage of samples showed altered concentrations in the compound classes of amino acids, acylcarnitines and phosphatidylcholines.

In a multicenter clinical trial, metabolic changes that were induced by single metformin intake or short-term therapy in newly diagnosed type 2 diabetes patients were analyzed. It was found that levels of metabolites within the urea cycle, in particular citrulline, and specific medium-chain acylcarnitines were altered after the first dose of metformin. After 4-6

weeks of treatment, levels of metabolites within the urea cycle, the ratio of hydroxylated to non-hydroxylated sphingomyelins, and the balance of branched chain amino acids to aromatic amino acids were affected by changes.

Overall, this thesis gives important information on the impact of pre-analytical influences on different metabolites and also provides new possible ways how metformin influences the human metabolome.

ZUSAMMENFASSUNG

Die präanalytische Phase einer klinischen Studie umfasst alle Schritte, die vor der Probenmessung stattfinden. Diese Phase ist am anfälligsten für Fehler und hat daher einen wichtigen Einfluss auf die Qualität und Validität der Studienergebnisse. Bisher gibt es nur wenige Informationen über den Einfluss der Präanalytik auf Metaboliten in humanen Proben. Zudem erschweren natürlich vorkommende Schwankungen der Metabolitkonzentrationen im Blut die Interpretation von Studienergebnissen.

In der vorliegenden Arbeit wurde die Stabilität verschiedener Metaboliten aus Serum- und Plasmaproben gesunder Menschen während der präanalytischen Phase unter dem Einfluss ausgewählter Parameter bestimmt. Basierend auf Flüssigchromatographie-Massenspektrometrie wurden Metabolitkonzentrationen aus sechs verschiedenen Stoffklassen mittels ‘targeted metabolomics‘ analysiert. Dabei konnten verlässliche und stabile Metaboliten identifiziert werden, welche für ‘targeted metabolomics‘ Analysen in multizentrische Studien geeignet sind. Metaboliten in Plasma und Serum zeigten eine hohe Verlässlichkeit in den Stoffklassen der Aminosäuren, biogenen Amine, gesättigten kurz- und mittelkettigen Acylcarnitine, Glycerophospholipide, Sphingolipide und der Summe der Hexosen. Mit Ausnahme von Methioninsulfoxid hatten Serumröhrchen mit Trenngel verglichen mit Standardröhrchen keinen Einfluss auf die untersuchten Metaboliten. Es wurde zudem festgestellt, dass während der Transportsimulation und bei bis zu zwei Auftauzyklen die meisten Metaboliten stabile Konzentrationen aufwiesen. Des Weiteren wurde eine Methode zur Evaluation der Tauglichkeit von Proben nach Langzeitlagerung für ‘targeted metabolomics‘ vorgestellt. In der hierfür untersuchten Studienkohorte konnten Veränderungen der Metabolitkonzentrationen in den Stoffklassen der Aminosäuren, der Acylcarnitine und der Phosphatidylcholine durch mehrjährige Probenlagerung bei -20°C detektiert werden.

In einer multizentrischen, klinischen Studie wurden Veränderungen des Metaboloms in Patienten mit neu diagnostiziertem Typ 2 Diabetes untersucht, welche durch einmalige Gabe oder kurzzeitige Therapie mit Metformin hervorgerufen wurden. Konzentrationsänderungen von Metaboliten innerhalb des Harnstoffzyklus, insbesondere von Citrullin, und bestimmter mittelkettiger Acylcarnitine traten nach der ersten Metformindosis auf. Nach vier- bis sechswöchiger Metforminbehandlung wurden Konzentrationsänderungen in Metaboliten des Harnstoffzyklus, dem Verhältnis von verzweigtkettigen zu aromatischen Aminosäuren und dem Verhältnis von hydroxylierten zu nicht-hydroxylierten Sphingomyelinen gefunden. Insgesamt stellt diese Arbeit wichtige Informationen über die Auswirkungen präanalytischer Einflüsse auf verschiedene Metaboliten dar und zeigt zudem neue mögliche Wege auf, wie sich die Einnahme von Metformin auf das humane Metabolitprofil auswirkt.

ABBREVIATIONS

aa diacyl

AAA aromatic amino acids

ADA American Diabetes Association

ADMA asymmetric dimethylarginine

ae acyl-alkyl

alkyl-DHAP alkylglycerone phosphate synthase

alpha-AAA alpha-aminoapidic acid

AMPK adenosine monophosphat-activated protein kinase

ATP adenosine triphosphat

BCAA Branched chain amino acids

BCVs Between-person coefficients of variance

C10:1 decenoylcarnitine

C5-M-DC Methylglutarylcarnitine

C8 octanoylcarnitine

CI confidence interval

Cit citrulline

CP cool packs

CV coefficient of variance

DMA dimethylarginine

FA2H fatty acid 2-hydroxylase

HbA_{1c} glycated hemoglobin

ICC intraclass correlation coefficient

IDF International Diabetes Federation

KORA Cooperative Health Research in the region of Augsburg

LC/MS-MS liquid chromatography-tandem mass spectrometry

lysoPCs lysophosphatidylcholines

Met-SO Methionine sulfoxide

MS mass spectrometry

NMR nuclear magnetic resonance

NOS nitric oxide synthase

OGTT oral glucose tolerance test

Orn ornithine

OTC Ornithine transcarbamoylase

PCs phosphatidylcholines

RT room temperature

SDMA symmetric dimethylarginine

SM sphingomyelins

T2DM type 2 diabetes mellitus

total SM-OH/total SM-non-OH ratio of hydroxylated to non-hydroxylated sphingomyelins

WCVs within-person coefficients of variance

1. INTRODUCTION

1.1 METABOLOMICS

1.1.1 Definitions

Metabolites are small molecules with an atomic mass of less than 1 kDa and are intermediates or products of metabolism [1]. ‘Metabolism’ originates from the Greek word metabolē 'change' (from metaballein 'to change') and the suffix –ism [2] and describes the chemical processes which occur within an organism. The total number of metabolites present within an organism refers to the ‘metabolome’. Each of the 500 different cell types of the human body has specific functions and therefore a different gene expression pattern, proteome and metabolome [3]. On the pattern of other omics studies (e. g., genomics, proteomics), the terms ‘metabolomics’ and ‘metabonomics’ were introduced in the late 1990s [3,4]. Both terms have been used in the context of the measurement of metabolites, but the classification of their usage is indistinct. Whereas ‘metabolomics’ puts its stress on studies of metabolites produced by the host organism, ‘metabonomics’ is defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [4]. However, often both terms are applied synonymously [3]. At the moment, the measurement of metabolic profiles is mainly performed with either mass spectrometry (MS) or nuclear magnetic resonance (NMR) techniques, while both techniques use fundamentally different methods of analyte quantification.

MS metabolic profiling can be performed in targeted or untargeted mode. Targeted metabolomics provides quantitative data on a predefined set of molecules and is a more hypothesis-driven approach. Untargeted metabolomics analysis is performed to measure as

many metabolites as possible in a biological sample, but only semi-quantitative data in terms of ion counts are provided [5].

1.1.2 Applications

The metabolic profile of an individual reflects its current metabolic state [6]. Besides other factors, the metabolic profile is influenced by disease status and medication [1,7]. Thus, altered metabolite levels could be utilized as diagnostic biomarkers that reveal the status of a disease process [8,9] and might facilitate preventive actions in early stages of several diseases [10]. Risk stratification on the basis of metabolic profiles and the identification of individuals most at risk of a certain disease can even help to take preventive measures before disease onset [11]. In the ancient Greece it was already well known that changes in tissues and biological fluids are indicative of diseases. So called ‘urine wheels’ were used since the Middle Ages in order to diagnose nephrology and other diseases according to the color, taste and smell of urine [12,13]. Thus, over the last centuries, applications of metabolomics have substantially contributed to improving human health and to reducing health care costs.

Up to now, a multitude of markers for different diseases have been investigated [12,14-18] and the discovery of novel metabolic biomarkers is further used to identify new biochemical pathways and targets for drug design [12]. The monitoring of drug efficacy and detailed insights into the mechanisms of drug action with means of metabolomics analyses help to develop tailored therapies and to improve application in theranostics [19], also known as personalized medicine [1,20]. There is a great potential for the future application of anti-infectives from microorganisms as metabolomics provide the opportunity to investigate biodiversity by screening less studied taxa and habitats or bacteria which are difficult to culture [20,21]. This screening also helps to improve the understanding of the impact of gut

microbes on human health [22]. Further applications of metabolomics are the measurement of food quality and determination of food origin [11].

1.2 THE PRE-ANALYTICAL PHASE

1.2.1 Definition

Clinical trials are usually divided into three major phases: the pre-analytical, the analytical and the post-analytical phase. The pre-analytical phase includes six major steps:
1) patient identification, 2) patient preparation, 3) sample collection, 4) transport and handling, 5) storage, and 6) preparation for testing [23].
The analytical phase involves the measurement of samples on a platform and the post-analytical phase includes all steps in a clinical trial after data collection is completed.

1.2.2 Variation of metabolic profiles in humans

In clinical trials, often only one blood sample is collected from each participant [24] and a single time point measurement is supposed to represent the metabolic status of an individual sufficiently [25]. Metabolite levels in humans are influenced by several factors like genetic background [26,27], sex [28], age [29], diet [30], gut microbiome, physical activity, hormone- and stress levels [1,31,32], and circadian rhythm [33]. For this reason, intra- and interindividual variations also occur in healthy humans [34-37]. It might be difficult to distinguish between changes in metabolite levels which result from disease-related processes and changes induced by normal physiological variation [32], also called in vivo pre-analytical variation [38]. This uncertainty leads to reduced power of a study [39] and might limit the usability of the analyzed metabolites as biomarkers. It is therefore important to evaluate the reliability of new potential biomarkers before their usage in clinical application.

To obtain reliable results by a single blood measurement, the within-subject variance over time should be small compared to the between-subject variance [24,40]. To measure the reliability of an analyte, the intraclass correlation coefficient (ICC) was introduced by Shrout and Fleiss [41]. It is calculated as between-subject variance divided by the sum of between- and within-subject variance [41]. Floegel et al. [42] suggested a classification of the ICC into four groups (i. e., ICC ≥ 0.75: excellent reliability; 0.50 < ICC < 0.75: good reliability; 0.40 ≤ ICC ≤ 0.50: fair reliability; ICC < 0.40: poor reliability). Besides the in vivo pre-analytical variation, other variations of the metabolic profile which might occur during the pre-analytical phase have to be considered.

1.2.3 Relevance of errors during the pre-analytical phase

The pre-analytical phase is much more vulnerable to errors than all other steps during the whole testing process [43,44] (FIGURE 1). In this context, an error is defined as any mistake during the entire process which somehow influences the quality of the laboratory outcome [45].

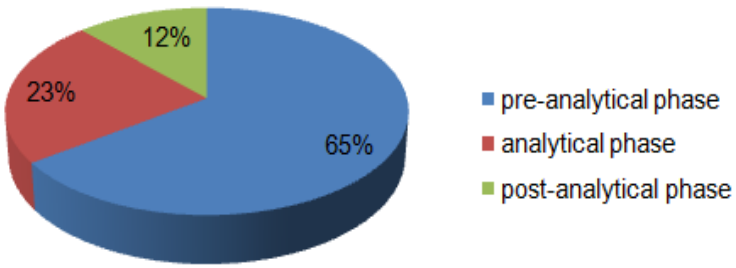

FIGURE 1. Distribution of errors that occur in clinical trials (numbers from [44]).

Errors are mainly introduced in the pre-analytical phase of a clinical trial.

Because of their major implications on the quality of test results, the prevention of pre-analytical errors has been an important goal during the last decades [46]. In 1981, the National Committee for Clinical Laboratory Standards (now Clinical and Laboratory

Standards Institute) introduced pre-analytical standards [47]. Despite these actions, much more progress was achieved in improving analytical quality and so far, no commonly used procedures for the identification and handling of unsuitable samples and the documentation of aberrations have been developed and implemented satisfactorily [23,48].

During the pre-analytical phase, common errors like the usage of the wrong container, hemolysis, clotting, insufficient sample volume, contamination, and misidentification of samples might arise [49]. However, little information is available on errors concerning changes in analyte concentrations caused by interactions with materials of sample collection tubes, transport delays, repeated freezing and thawing and long-term storage under different conditions. The extent of changes in analyte concentrations, which are caused by errors during the pre-analytical phase, depends on the sensitivity of the investigated compound [50]. Thus, the success of a study which includes the measurement of susceptible analytes is highly dependent on the quality of the analyzed samples and on the control of parameters which introduce an error during the pre-analytical phase [51]. Especially for the increasing number of biobanks, it is important to have knowledge on the suitability of the stored specimen for specific downstream applications, for example metabolomics measurements [38].

1.2.4 Suitability of gel-barrier collection tubes

At the beginning of the sample collection procedure, the choice of a suitable collection tube has to be made. Besides standard serum tubes with clotting activator, tubes which contain a separator gel have been in clinical use for several decades. The gel inside the tubes separates the serum from clotted whole blood [52] and usually consists of acrylic, silicone or polyester polymers [53]. By a temporary change in viscosity during centrifugation, the gel locates between the cells and the overlying serum layer [54].

Advantages of serum gel-barrier tubes over standard tubes are the ease of handling, the necessity of only one centrifugation step and therefore, reduced processing time, a higher serum yield, and reduced aerosolization of hazardous substances [55]. Besides, also the separator gel can be a source of pre-analytical errors [53,54,56-59]. Adsorption of analytes from specimen into the separator gel and elusion of materials from the gel might interfere with different assays and was reported previously [53,56,57,60-63]. As the separator gels are considered hydrophobic, there is a higher risk for adsorption of lipophilic analytes [53]. Until now, a limited number of studies investigated the effect of serum separator gels on different metabolites and only standard laboratory parameters like creatinine and glucose were analyzed [54,64].

1.2.5 Delay of sample transport

In clinical studies, there is a trend towards the decentralized collection of blood samples in individual facilities and transportation of samples to a central laboratory [54,65-67]. However, in multicenter studies immediate processing and transportation of samples after collection is impractical [68,69]. Therefore, it is of particular interest to assess which analytes show stable concentrations under delayed processing and transportation of samples [67,70].

Although several studies investigated the impact of sample handling and transportation under different conditions [24,27,54,67,69-83], information regarding metabolite stability is still limited. Metabolites which are sensitive to delayed processing and transportation can be used to control for sample quality [73]. Whereas, when metabolites are of interest that prove to be robust against deviation of guidelines more convenient and cost-efficient processing and transportation might be possible.

1.2.6 Repeated freezing and thawing

Like tube type and time- and temperature variation during sample handling and transportation, repeated freezing and thawing of specimen should be considered as a possible source of errors in the pre-analytical phase. As a general guideline, it is recommended to use samples which were frozen and thawed only once and to keep the number of freeze-thaw cycles consistent between the analyzed samples [37]. The feasibility of this guideline is hampered by restricted freezer space and the limited number of aliquots that can be collected from each person [84]. Consequently, the usage of repeatedly frozen and thawed samples is inevitable, if repeated measurements or additional analyses are performed [85]. Furthermore, additional aliquots for future applications are usually not provided. Thus, the evaluation of the impact of repeated freezing and thawing on the sample quality is of importance. Various studies examined the influence of multiple freeze-thaw cycles on the stability of analytes in different specimen [37,71,80,84-86] and showed only minor changes. However, less information on the stability of metabolites in human serum samples is available.

1.2.7 Long-term storage under different conditions

In order to maintain long-term integrity of biomarkers, it is recommended to store samples in freezers at -80°C or below [87]. However, millions of human biosamples are stored in biobanks established decades ago. These samples were collected and processed at a time when the pre-analytical phase was less standardized, and therefore they often do not meet the requirements of today's omics technologies [88].

As an increasing number of these cohorts will be analyzed in the future, the impact of different long-term storage conditions on analytes will become an important issue regarding the quality and credibility of study results. In particular, it is of interest whether the usability of

long-term stored samples would be problematic for targeted metabolomics analysis. In contrast to DNA and RNA, there is no standard method to evaluate the quality of metabolite measurements as each metabolite shows its unique stability profile [88]. During long-term storage, some metabolite concentrations might have changed systematically and the resulting instability of metabolites may lead to an increased pre-analytical variability [75]. Hence, the impact of long-term storage conditions on the metabolome is difficult to define comprehensively [88]. For the analysis of archived samples using new technologies, no comparable data exist as during sample collection these analysis methods haven't been developed [88]. Thus, new ways of sample evaluation need to be developed to estimate the reliability of omics data obtained from long-term stored samples.

1.3 TYPE 2 DIABETES

1.3.1 Definition and Prevalence

The term 'diabetes mellitus' summarizes a group of chronic metabolic diseases which is characterized by hyperglycemia. Diabetes results either by progressive failure of pancreatic β-cell function and a consequent lack of insulin production (type 1 diabetes mellitus), or by the development of insulin resistance and the subsequent loss of β-cell function (type 2 diabetes mellitus, T2DM) [89,90]. Based on the pathogenesis of the disease, two other classes of diabetes were defined by the American Diabetes Association (ADA); namely gestational diabetes and 'other specific types of diabetes', which include genetic defects of β-cell function and insulin action [89].

However, T2DM is the most common type of diabetes and accounts for 85%-95% of all cases [91]. Usually appearing in adults, T2DM progressively arises in children and adolescents [91]. Different subphenotypes of T2DM have been identified [92,93] which show the complexity of this disease and necessity of enhancement of T2DM subclassification in

order to improve individualized treatment [94]. Since the 1970s, the oral glucose tolerance test (OGTT) has been the standard tool for diagnosis of diabetes [95]. According to the ADA, the criteria for diagnosis of diabetes are 1) glycated hemoglobin (HbA_{1c}) ≥ 6.5%, or 2) fasting plasma glucose levels ≥ 126 mg/dL (7.0 mmol/L), whereas fasting is defined as no caloric intake for at least 8h, or 3) 2h plasma glucose ≥ 200 mg/dL (11.1 mmol/L) during an OGTT, or 4) a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) in patients with classic symptoms of hyperglycemia or hyperglycemic crisis [89].

In 2013, the International Diabetes Federation (IDF) estimated the worldwide diabetes prevalence at more than 8% (absolute numbers in FIGURE 2) and predicted an increase of 55% in the absolute number of diabetes cases by 2035. In 2035, approximately 592 million people worldwide will suffer from diabetes [91].

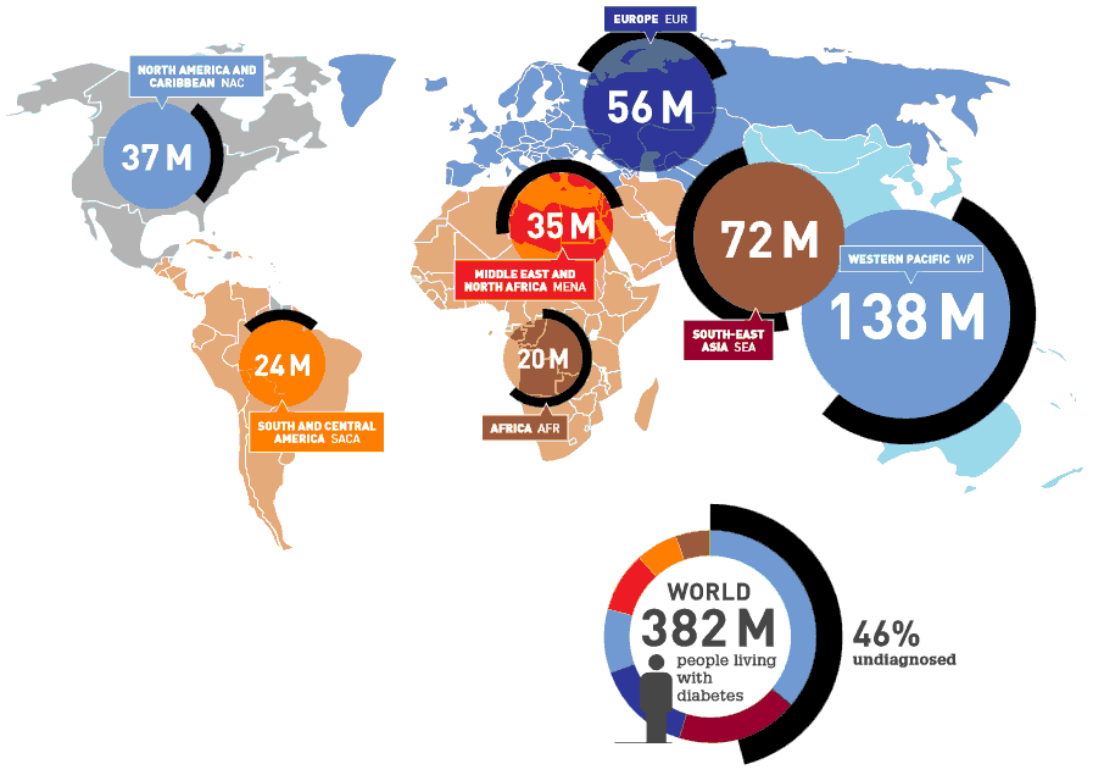

FIGURE 2. Absolute number of people with diabetes in 2013, divided according to IDF Region (published with permission from the IDF [91]).

Black borders represent portion of undiagnosed cases of diabetes. M; million.

1.3.2 Metabolomics in diabetes research

During the last decade, metabolomics have provided new insights into the pathology of diabetes and revealed methods to predict the course of disease [95]. However, there is a clinical need to identify biomarkers of insulin resistance and diabetes that are able to predict the disease before its manifestation [96].

Linoleoylglycerophosphocholine and a-hydroxybutyrate were shown to be early markers of insulin resistance [17,97]. In addition, alterations in urea cycle-related metabolites like citrulline (Cit), Asp, and N-acetylglutamate were reported to correlate with insulin resistance [98]. T2DM is associated with altered metabolite levels in carbohydrate metabolism and the tricarboxylic acid cycle [99-101], lipid metabolism [10,99,102-109], and certain amino acids [10,99-101,104,107-109]. Levels of 2-aminoadipic acid [16], branched chain amino acids (BCAA), and aromatic amino acids (AAA) [15] are linked to the risk of developing T2DM, but it is unknown whether alterations in BCAA metabolism play a causative role in pathogenesis or are outcomes of metabolic dysfunction [96,110]. It was found that the overall nutritional status of an individual mainly influences the potential to induce diabetes by BCAA supplementation [111] and that levels of several metabolites, mainly lipids, are highly dependent on nutrition [30], which makes it difficult to include these metabolites as biomarkers into routine diagnostics. However, metabolomics in the field of diabetes may provide biomarkers associated with different drug responses that could predict the efficacy of clinical interventions.

1.3.3 Treatment strategies against type 2 diabetes

The goal of an antidiabetic therapy is to reduce blood glucose and HbA_{1c} levels without causing hypoglycemia. As lifestyle, including unhealthy diet, insufficient physical activity, and factors like overweight or obesity, and stress, are the main causes of T2DM, the initial

therapeutic approach includes lifestyle changes, such as change of diet, regular physical activity, loss of body weight and smoking cessation [95]. If this approach is not successful, a drug-based therapy follows. The most frequently applied drug for the therapy of T2DM is metformin [112]. Other commonly prescribed antidiabetic drugs are insulin secretagogues (sulfonylureas) [113], and insulin sensitizing peroxisome proliferator-activated receptor gamma agonists (thiazolidinediones) [114].

1.4 METFORMIN

1.4.1 Application and contraindication

Metformin is recommended by the ADA as the standard first-line oral antihyperglycemic agent and is one of the few drugs available to pharmacologically reduce insulin resistance in T2DM patients. It has been in clinical use for more than 50 years [112]. Metformin belongs to the class of biguanides (FIGURE 3).

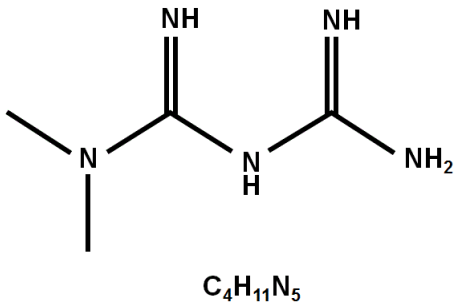

FIGURE 3. Structural formula of metformin.

Clinical responses to metformin are variable. It cannot be applied in patients with renal insufficiency, because of a risk of lactic acidosis, which represents a very rare but life-threatening complication of metformin use [115,116]. Furthermore, about 20% of patients suffer from gastrointestinal side effects of metformin, which often leads to the discontinuation of treatment [117].

1.4.2 Mode of action

It is believed that the primary target for metformin is complex I of the mitochondrial respiratory chain [118]. Metformin impairs adenosine triphosphat (ATP) production and activates adenosine monophosphat-activated protein kinase (AMPK). A decrease in energy charge enhances glycolysis and glucose uptake into cells and increases oxidation of fatty acids to restore the energy balance, whereas energy demanding processes such as hepatic glucose production and lipid and cholesterol synthesis are inhibited [119-121]. However, there is recent evidence that the inhibition of complex I by metformin also leads to an AMPK-independent reduction in cellular energy charge [119]. The molecular nature of these actions is unknown.

1.5 AIMS OF THIS THESIS

The overall goal of the present thesis was the determination of the in vivo- and in vitro pre-analytical stability of metabolites that are of special interest in multicenter clinical trials and the investigation of drug action on the human metabolome with focus on T2DM.

The first objective was to assess the within- and between subject reproducibility of targeted metabolomics measurements, as metabolite levels in an individual need to be adequately stable over time to allow for the measurement of changes provoked by an intervention.

A substantial amount of clinical trials are multicenter studies and therefore, the validation of realistic and cost-effective ways of pre-analytical sample handling is of particular importance. To incorporate metabolomics into such studies, the second aim was to evaluate the impact of different steps during the pre-analytical phase, such as

1) choice of tube type,

2) transportation,

3) repeated freeze-thaw cycles,

and 4) long-term storage on the stability of metabolites.

T2DM is an epidemic of the 21st century and requires global attention, as the number of cases increases tremendously every year. The mode of action of metformin, the most commonly prescribed drug against T2DM, is still unknown. Therefore, the third aim was to determine drug-induced changes in the metabolome of T2DM patients in order to gain new insights into the mechanisms of metformin action and also into potential sex-specific effects of this drug.

2. MATERIAL AND METHODS

2.1 SAMPLE COLLECTION AND PREPARATION

2.1.1 Reliability of plasma and serum metabolites

Blood samples were collected from 20 healthy volunteers (5 males and 15 females) in Munich, Germany. Participants had a mean age of 30 (range: 22-52). Samples were collected after overnight fasting in 7.5 mL safety-monovettes (Sarstedt, Nümbrecht, Germany). Information regarding medication and the last meal before each fasting period was recorded for each sampling day. All participants were non-smokers. For preparation of plasma ('plasma baseline'), the K^+EDTA^--monovettes were centrifuged immediately (2000 x g, 10min). One monovette for serum preparation ('serum baseline', with clot activator) was centrifuged after 30min of coagulation at room temperature (RT) (~21°C). Serum baseline and plasma baseline samples were stored as 0.25 mL aliquots on dry ice and frozen at -80°C before measurement. The baseline samples were collected on three different days from the same individuals within a time period of 14 days.

2.1.2 Effect of serum gel-barrier tubes on metabolites

From 15 healthy participants (four males, eleven females, see 2.1.1.) blood was collected in a 7.5 mL serum tube for serum W and a 7.5 mL serum gel-barrier tube containing a polyacrylester gel, respectively (Sarstedt, Nümbrecht, Germany). Both tube types contained clot activator. For a subgroup of five volunteers (1 male and 4 females) a second set of serum W and serum gel-barrier samples was collected during an additional collection day. Aliquots of 0.25 mL of serum W tubes were frozen at -20°C after centrifugation. Whole

serum gel-barrier tubes were frozen immediately at -20°C after centrifugation. Tubes were stored at -80°C without additional thawing before measurement.

2.1.3 Effect of simulated shipment on metabolites

Blood samples were collected from the same 20 participants as in 2.1.1. Serum baseline and plasma baseline samples were prepared and stored as in 2.1.1. Three serum tubes (serum gel-barrier tubes with clot activator) were stored horizontally on cool packs (CP) (~4°C) for 3, 6 and 24h before centrifugation. In addition, four plasma tubes from each person were stored horizontally on CP for 3, 6 and 24h and one tube at RT for 24h before centrifugation. Aliquots of 0.25 mL plasma and serum were stored at -80°C before measurement.

2.1.4 Effect of freeze-thaw cycles on serum metabolites

Blood was collected from the same 15 participants as in 2.1.2. From a subgroup, a second set of serum gel-barrier samples was collected (see 2.1.2). Three 7.5 mL serum gel-barrier tubes were prepared. One serum gel-barrier tube from each person was frozen immediately at -20°C and stored at -80°C without additional thawing before measurement (serum gel control). Aliquots of 0.25 mL of the remaining two serum gel-barrier tubes were frozen at -20°C and thawed once and twice at RT after one week, respectively, before storage at -80°C.

2.1.5 Impact of long-term storage on metabolites

Samples from 13 healthy male participants were randomly selected from a Danish cohort from the University of Copenhagen. For clinical characteristics of this subgroup see TABLE 1.

TABLE 1. Clinical characteristics of selected individuals from a Danish cohort.

	baseline	follow-up
Sample size	13	13
Age* (years)	32 ± 1	43 ± 1
BMI* (kg/m^2)	25.4 ± 2.2	26.8 ± 2.3
HbA_{1c}* (%) (mmol/mol)	5.6 ± 0.2 (38.2 ± 2.7)	5.3 ± 0.2 (34.1 ± 2.7)

*Mean ± SD

Plasma baseline samples were collected at the beginning of an intravenous glucose tolerance test or in one case at the beginning of an OGTT between 1993 and 1996. Follow-up samples were taken at the beginning of an OGTT between 2005 and 2006. All plasma samples were stored at -20°C in tubes from different manufacturers. No information is available on the number of freeze-thaw cycles or on varying sample-storage conditions. Before liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement, each sample was divided into three aliquots to calculate the technical variance of the measurement.

2.1.6 Metabolic changes induced by metformin

Study population

Study participants were recruited through primary care physicians in the state of Bavaria, Germany. Inclusion criteria were diagnosed T2DM, no antidiabetic drug therapy for at least three months prior, and a decision by the treating local physician to start metformin monotherapy. The study design was prospective and purely observational. All data and samples were collected at the offices of the local physicians, and therapeutic decisions were made at the discretion of the treating physicians. The characteristics of the study populations are shown in TABLE 2.

TABLE 2. Clinical characteristics of the individuals in the discovery and replication studies.

	Discovery study	Replication study
Sample size (male/female)	30 (21/9)	164 (82/82)
Age* (years)	59 ± 10	59 ± 13
BMI* (kg/m^2)	34 ± 6	32 ± 6
HbA_{1c}* (%) (mmol/mol)	7.3 ± 1.7 (56.2 ± 18.3)	7.2 ± 1.4 (55.1 ± 15.6)

*Mean at baseline ± SD

Sample collection and preparation

Serum samples were collected from newly diagnosed T2DM patients after an overnight fast (discovery study *n* = 30, replication study *n* = 164; time point A). The first dose of metformin (500-1000 mg) was administered to each study participant between 6:00 and 9:00 PM within the following three days. The second fasting serum sample was collected the following morning, 10–16h after the first dose of metformin (time point B). A third fasting serum sample was collected from a subgroup of 19 patients in the discovery study (11 males and 8 females) and 117 patients in the replication study (60 males and 57 females) 4–6 weeks after the initiation of metformin monotherapy (time point C). After individualized dose titration, the daily dose of metformin at time point C was between 1000 and 2000 mg. All blood samples were collected in 7.5 mL serum gel-barrier tubes (Sarstedt, Nümbrecht, Germany). Blood was allowed to coagulate for 30min at RT. Samples from sites outside of Munich were centrifuged on-site, frozen at -20°C and transported to the central laboratory on dry ice within four weeks. Samples collected at sites in Munich were cooled down to 4°C, transported to the central laboratory within 2-6 h, centrifuged and separated in aliquots. In the central laboratory, serum samples were stored at -80°C until analysis.

2.2 METABOLITE ANALYSIS

The targeted metabolomics approach was based on electrospray ionization LC-MS/MS and MS/MS measurements using the Absolute*IDQ*^{TM} p180 kit (BIOCRATES Life Sciences AG,

Innsbruck, Austria). The assay allowed simultaneous quantification of 188 metabolites in 10 µL of serum and plasma, including free carnitine, 40 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic amino acids, Cit and ornithine [Orn]), 21 biogenic amines, hexose (sum of hexoses–approximately 90%–95% glucose), 90 glycerophospholipids (14 lysophosphatidylcholines [lysoPCs] and 76 phosphatidylcholines [PCs] [diacyl {aa} and acyl-alkyl {ae}]), and 15 sphingomyelins (SMx:y). The abbreviation Cx:y is used to describe the total number of carbons (x) and double bonds of all chains (y) (as described in [122]). The Absolute*IDQ*™ p180 kit conforms with the Food and Drug Administration "Guidance for Industry: Bioanalytical Method Validation" [123], which implies proof of reproducibility within a given error range. Measurements were performed as described in the manufacturer's manual UM-P180. The assay procedures of the Absolute*IDQ*™ p180 kit and the metabolite nomenclature were described in detail previously [122,124]. Sample handling was performed using a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK). MS analyses were conducted on an API 4000 LC-MS/MS System (AB Sciex Deutschland GmbH, Darmstadt, Germany) equipped with 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Boeblingen, Germany) and HTC PAL auto samplers (CTC Analytics, Zwingen, Switzerland) and controlled by Analyst 1.5.1 software. For the calculation of metabolite concentrations, internal standards served as a reference. Concentrations of all metabolites were calculated in µmol/L. The analytical variance was determined by measuring metabolite concentrations of a reference sample of pooled human plasma with five replicates on each plate. In addition to the 188 absolute metabolite concentrations, 44 predefined sums and ratios from the MetaDis*IDQ*™ kit (BIOCRATES) were calculated using Met*IDQ*™ software (manufacturer's manual BB-MD-1). Absolute metabolite concentrations and predefined sums and ratios are referred to as metabolite traits. A list of all investigated metabolite traits is given in SUPPLEMENTARY TABLE 1.

2.3 DATA PREPROCESSING AND QUALITY CONTROL

Data preprocessing and quality control were performed separately in each study cohort. All statistical analyses were performed with the statistic platform R, versions 2.14.1 and 3.0.2 [125]. A plate correction was performed by multiplying all metabolite concentrations with a correction factor for each plate (geometric mean of the geometric means of the reference plasma samples across all plates divided by the geometric mean of the reference plasma samples of each plate). Metabolites were excluded from further analysis, if they did not pass quality control. Exclusion criteria were: 1) more than 50% of measurements at zero concentration or missing, 2) concentrations below the limit of detection in more than 95% (for Danish study cohort: in more than 50%) of all samples. If the coefficient of variance (CV) across all plates was ≥ 25% in the reference samples, the metabolite was labeled (*), as it may have a lower CV across all plates in further investigations. In the Danish study cohort, metabolites with CV≥ 25% in the reference samples were excluded.

2.4 STATISTICAL ANALYSIS

2.4.1 Reliability of plasma and serum metabolites

Coefficient of variance

Between-person coefficients of variance (BCVs) and within-person coefficients of variance (WCVs) and between- and within-plate CVs were calculated as the square root of the between- and within-subject/plate variances as determined using random effects models (R package *nlme*) divided by the mean metabolite concentration. The median within-plate CV was 9.31% and the median between-plate CV was 1.74E-04%.

Intraclass correlation coefficient

Reliability was expressed as the ICC, calculated as between-subject variance divided by the sum of between- and within-subject variances [41]. Bias corrected and accelerated bootstrap confidence intervals (CI) were calculated for BCVs and WCVs and the ICC using 10,000 bootstrap samples [126].

Metabolite levels in serum and plasma

Significant differences in metabolite concentrations between serum baseline and plasma baseline samples were tested in a pairwise manner using Wilcoxon signed-rank tests. The level of statistical significance was set to $p < 0.01$.

2.4.2 Effect of serum gel-barrier tubes on metabolites

The effect of tube type (serum W tube vs. serum gel-barrier tube) on metabolite concentrations was analyzed using non-parametric Friedman tests for each metabolite. The level of statistical significance was set to $p < 0.01$.

2.4.3 Effect of simulated shipment on metabolites

Effect of time and temperature on metabolites

In order to explore time and temperature effects on metabolite concentrations in plasma and serum samples during simulation of transport, non-parametric Friedman tests were performed [127]. Significant changes in concentration were afterwards tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests. The level of statistical significance was set to $p < 0.01$.

Metabolite ratios

In addition to 158 absolute metabolite concentrations, all possible pairs of metabolite ratios (158*157/2=12403) were calculated as the natural logarithm of the quotient of two metabolite concentrations comparing plasma baseline samples and plasma samples which were stored at RT for 24h. Changes in metabolite ratios were determined using Wilcoxon signed-rank tests. For the metabolite ratios, a significance threshold of $p = 4.03E-06$ (Bonferroni-corrected for multiple comparisons) was used. The p-gain was utilized as an additional significance measure [99]. The critical value for the p-gain was set to ten times the number of investigated metabolites (p-gain > 1580) [128].

Power analysis

A power analysis was performed for each metabolite for selected settings (0h vs. 24h RT and 24h CP, respectively). The minimum concentration change between two paired settings was calculated for a power of 80% to recognize it as significant, at the given sample size of 20 and at an alpha level of 0.01. To account for the reduced power of the non-parametric Wilcoxon signed-rank test as compared with a parametric paired t-test, an asymptotic relative efficiency of 0.955 was assumed [129]. Results are given in SUPPLEMENTARY TABLE 2.

2.4.4 Effect of freeze-thaw cycles on serum metabolites

Non-parametric Friedman tests were performed to identify significant changes in metabolite concentrations between serum samples which were thawed 0x, 1x and 2x before measurement. Significant changes in concentration during the Friedman tests were afterwards tested in a pairwise manner against the reference (0x thawed) with Wilcoxon signed-rank tests. The level of statistical significance was set to $p < 0.01$.

2.4.5 Impact of long-term storage on metabolites

After the selection of a subset of samples which appropriately represent the investigated study cohort, the following steps were performed to evaluate sample quality and suitability for metabolomics measurements: 1) calculation of the technical CV from technical triplicates, 2) comparison of the sample groups (baseline and follow-up) with a defined reference range.

Technical variance

Each sample was measured in technical triplicates. For one sample, one technical replicate showed lower metabolite concentrations compared with the other two replicates, indicating a pipetting error. Therefore, this replicate was removed from further analysis. The technical CV for the targeted metabolomics measurement was calculated for 139 metabolites as the standard deviation divided by the mean metabolite concentration of the triplicates on the plate.

Comparison with reference range

A reference range for 116 metabolites was defined by using the range between the 2.5 percentile and the 97.5 percentile (95% reference interval) of metabolomics data from 1452 healthy males from the Cooperative Health Research in the region of Augsburg (KORA) F4 study [28]. Samples of the KORA F4 study were taken between 2006 and 2008. Until analysis with Absolute*IDQ*^{TM} Kit p150 (BIOCRATES), samples were stored at -80°C. Data of metabolites from the Danish study cohort were compared to the reference range. A sample was defined as “out of range” if the median metabolite concentration of technical replicates was above or below the reference range. Afterwards, McNemar test was performed for each metabolite in order to detect significantly higher proportions of baseline samples that were defined as “out of range” compared with the respective follow-up samples. The level of statistical significance was set to $p < 0.01$.

McNemar test statistic:

$$X^2 = \frac{(b-c)^2}{b+c}$$

(c): the baseline sample of an individual was out of range and the follow-up sample was within the reference range

(b): the baseline sample of an individual was within the reference range and the follow-up sample was out of the reference range

2.4.6 Metabolic changes induced by metformin

Changes in metabolite traits between time point A (before the first dose of metformin) and time point B (10–16h after the first dose) and between time point A and time point C (after 4–6 weeks of metformin therapy) were determined using linear mixed-effects models (R package *nlme*, version 3.1-113 [130]). Time point, sex, baseline HbA_{1c}, and age were modeled as fixed effects and sample identification as a random effect, and a two-sided t-test for zero time point effect was performed. A significance threshold of p = 5.95E-05 was used for the discovery study (Bonferroni-corrected for multiple comparisons). To investigate the presence of significant sex-specific effects, an interaction analysis was performed, in which sex*time point, sex*HbA_{1c}, and sex*age interactions were included as additional terms in the above described model. Significant sex*time point interaction was defined at p < 5.95E-05.
Because of the small sample size of the discovery study, metabolite traits with a nominally significant (p < 0.05) change in either time point comparison were put forward to a replication step, using a significance threshold of p = 2.78E-03 for the overall analysis and p = 7.14E-03 for the interaction analysis (Bonferroni-corrected for multiple comparisons). To ensure that the observed significant associations were robust against violation of the distribution assumptions of the model, bias corrected and accelerated bootstrap CI were additionally calculated using 4,000 bootstrap samples [126].

3. RESULTS

3.1 RELIABILITY OF PLASMA AND SERUM METABOLITES

Plasma and serum samples taken from 20 healthy human subjects on three different days were used to calculate the intraclass correlation coefficient (ICC), the within-person coefficient of variance (WCV) and the between-person CV (BCV) for 158 metabolites. SUPPLEMENTARY TABLE 3 summarizes results for ICC, WCV and BCV. Results are examined in more detail below.

3.1.1 Coefficients of variance

In plasma and serum metabolites, 80% of WCV and 70% of BCV values were below 0.25 (FIGURE 4). In plasma, the median WCV was 0.15 (range: 0.04–1.61) and the median BCV was 0.20 (range: 0.00–2.20). Median WCV values of metabolite subclasses were concordant in plasma and serum samples, whereas median BCV values were slightly higher in serum samples.

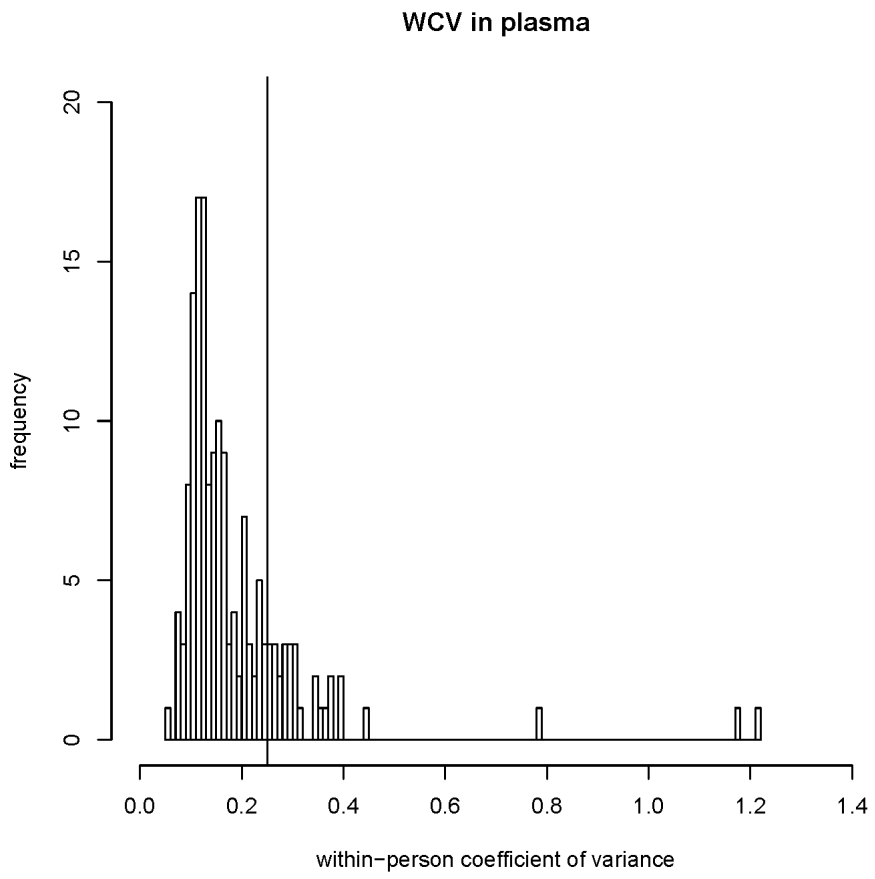

FIGURE 4. Histogram of WCV in plasma with flag at CV = 0.25.

3.1.2 Intraclass correlation coefficient

Both within- and between-person variance contribute to the ICC, a measure of the reliability of a metabolite. Accordingly, the median ICC was higher in serum as compared with plasma samples. The median ICC in plasma was 0.63. The lowest reliability in plasma was detected for PC ae C30:1* (ICC = 2.28E-09, CI: 0.00–0.47) and the highest for creatinine (ICC = 0.90, CI: 0.84–0.95). FIGURE 5 displays the investigated metabolites in plasma samples ordered by ICC value. In serum samples, the median ICC was 0.66. The lowest reliability in serum was observed for asymmetric dimethylarginine (ADMA) (ICC = 0.05, CI: 0.00–0.38) and the highest for spermidine* (ICC = 0.87, CI: 0.77–0.93).

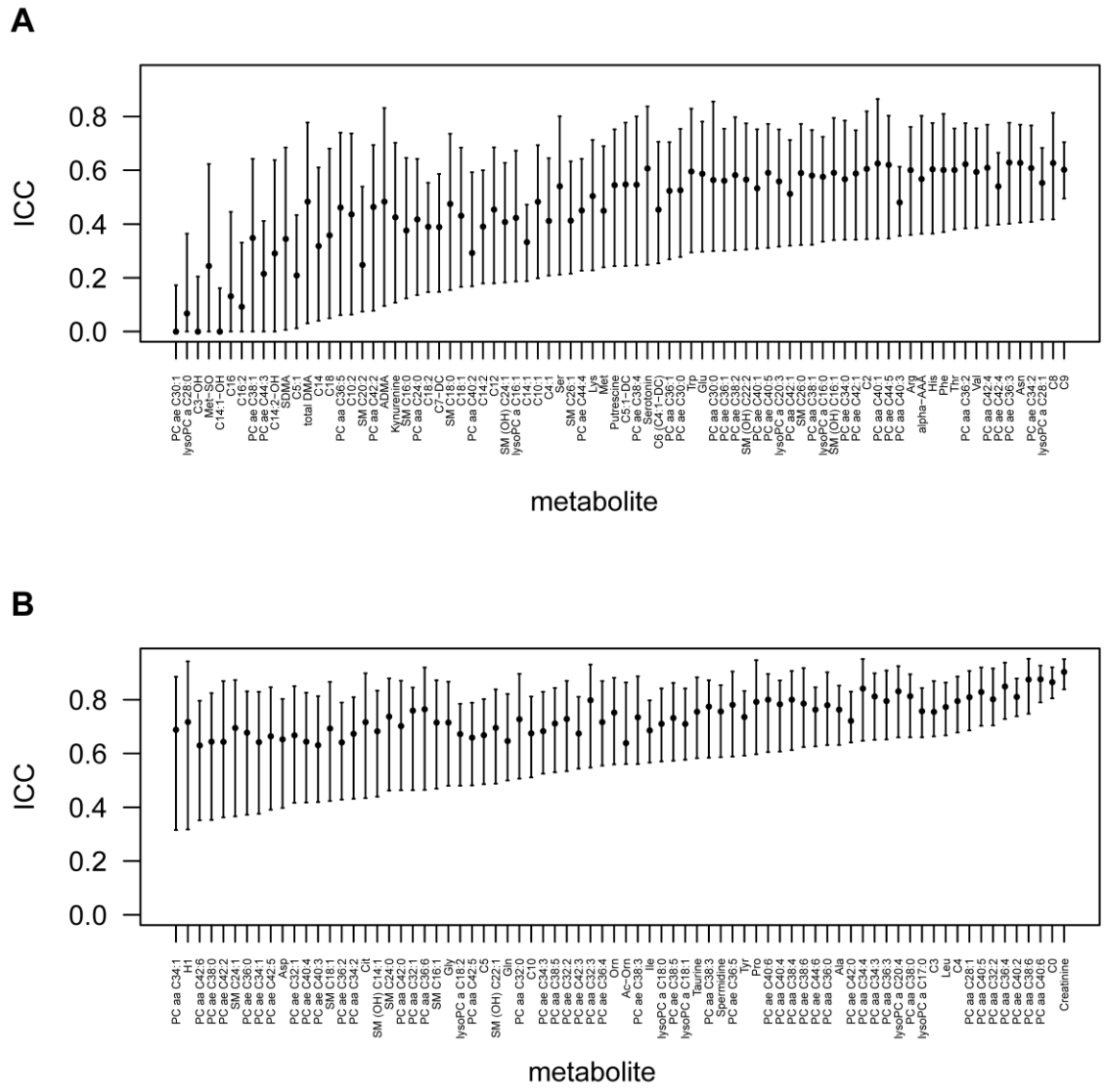

FIGURE 5. Median intraclass correlation coefficients (ICC) and confidence intervals of plasma metabolites.

(A) Median ICC values < 0.63 and (B) median ICC values ≥ 0.63 are displayed.

Regarding metabolite subclasses, reliability was lowest for acylcarnitines both in plasma and serum. All other subclasses showed good (ICC > 0.50) reliability both in plasma and serum samples.

3.1.3 Metabolite levels in plasma and serum

Significantly higher median concentrations in serum baseline compared with plasma baseline samples were shown for 101 metabolites (SUPPLEMENTARY TABLE 4). No effects of sex and last meal composition before each overnight fasting on metabolite concentrations were detected (data not shown).

3.2 EFFECT OF SERUM GEL-BARRIER TUBES ON METABOLITES

Especially in multicenter studies, the use of serum gel-barrier tubes has several advantages over standard tubes. Here, possible interactions of the gel with metabolites in human serum samples were analyzed. Blood was drawn into serum gel-barrier tubes and into serum W tubes (standard tubes which served as control). Both tube types contained clotting activator. After centrifugation, whole gel-barrier tubes and separated serum from serum W tubes were frozen for a few weeks at -80°C until analysis.

Methionine sulfoxide (Met-SO) showed significantly increased concentrations in serum gel-barrier tubes as compared with serum W tubes (FIGURE 6). For all other investigated metabolites, serum gel-barrier tubes had no significant effect on concentration compared with serum W tubes.

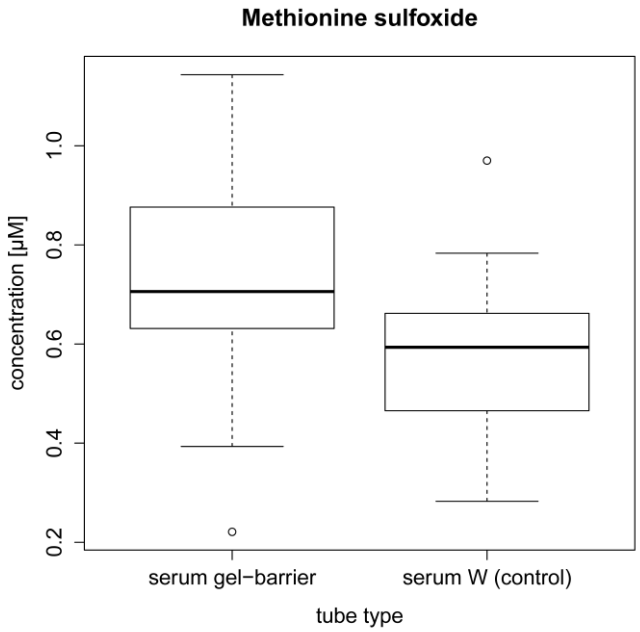

FIGURE 6. Effect of tube type on methionine sulfoxide in serum samples.

Significant difference in concentration between Met-SO in serum W tubes (control) and serum gel-barrier tubes were detected (Friedman test, significance level $p < 0.01$).

3.3 EFFECT OF SIMULATED SHIPMENT ON METABOLITES

3.3.1 Effect of simulated shipment on plasma metabolites

Transport of non-centrifuged samples on CP within 3, 6 and 24h (plasma and serum) or at RT for 24h (plasma only) was simulated to analyze different ways of sample collection and shipment which might occur in multicenter clinical trials.

In plasma samples, 44 out of 158 metabolites showed significant concentration changes between baseline (0h) and any of the other settings (TABLE 3) as determined by Friedman tests ($p < 0.01$).

TABLE 3. Impact of simulated shipment on metabolite concentrations in plasma samples.

Metabolites with significant changes in concentration after 3, 6 or 24h on cool packs (CP) and acceptable delay time for each metabolite during transportation (Wilcoxon signed rank test, comparison with baseline, significance level $p < 0.01$). For the displayed metabolites the acceptable delay time at RT was < 24h.

Metabolite	p-value (Friedman)	Acceptable delay time on CP
C10:2	3.32E-03	24h
C14*	2.16E-05	0h
C14:1-OH*	8.52E-04	24h
C16	1.87E-07	24h
C18*	4.45E-04	24h
C18:1	4.27E-13	3h
C18:2	3.61E-11	3h
C4:1	2.16E-05	24h
Ala	2.92E-08	6h
Arg	9.43E-14	0h
Asn	1.09E-05	24h
Asp	1.66E-11	6h
Glu	1.83E-10	6h
His	2.60E-04	24h
Ile	5.78E-03	24h
Leu	1.49E-07	6h
Lys	4.98E-07	6h
Met	3.54E-04	24h
Orn	1.12E-14	0h
Phe	7.54E-05	24h
Pro	1.15E-04	24h
Ser	9.74E-06	24h
Thr	8.41E-04	24h
Tyr	9.85E-04	24h
Acetylornithine	1.35E-05	24h
ADMA	3.53E-05	24h
alpha-aminoapidic acid (alpha-AAA)	3.49E-03	24h
Serotonin*	6.97E-07	3h
Spermidine*	7.66E-10	0h
Taurine	2.27E-10	0h
total DMA	1.50E-04	24h
lysoPC a C16:0	2.87E-09	24h
lysoPC a C16:1	2.44E-09	24h
lysoPC a C17:0	6.09E-08	24h

Metabolite	p-value (Friedman)	Acceptable delay time on CP
lysoPC a C18:0	6.55E-09	24h
lysoPC a C18:1	6.42E-09	24h
lysoPC a C18:2	1.26E-06	24h
lysoPC a C20:3	1.62E-07	24h
lysoPC a C20:4	2.84E-08	24h
PC aa 30:0	7.04E-04	24h
PC aa C32:1	7.43E-08	24h
PC aa C32:2	8.75E-06	24h
PC aa C34:3	6.64E-06	24h
Hexose	1.55E-14	3h

*Metabolites with CV across all plates ≥ 25% in reference samples

Concentrations of 145 plasma metabolites were stable for at least 24h on CP and 115 of these were also stable for at least 24h at RT. Of the 44 metabolites with significantly changed concentrations at RT, 35 showed increasing concentrations after 24h (SUPPLEMENTARY FIGURES 1-10) whereas nine showed decreasing concentrations, including C4:1 (SUPPLEMENTARY FIGURE 2), Arg (FIGURE 7), Met (SUPPLEMENTARY FIGURE 4), serotonin* (SUPPLEMENTARY FIGURE 7), four PC aa's (PC aa C30:0, PC aa C32:1, PC aa C32:2 and PC aa C34:3, SUPPLEMENTARY FIGURE 10) and the sum of hexoses (FIGURE 7). FIGURE 7 shows examples of significantly altered metabolite concentrations in plasma.

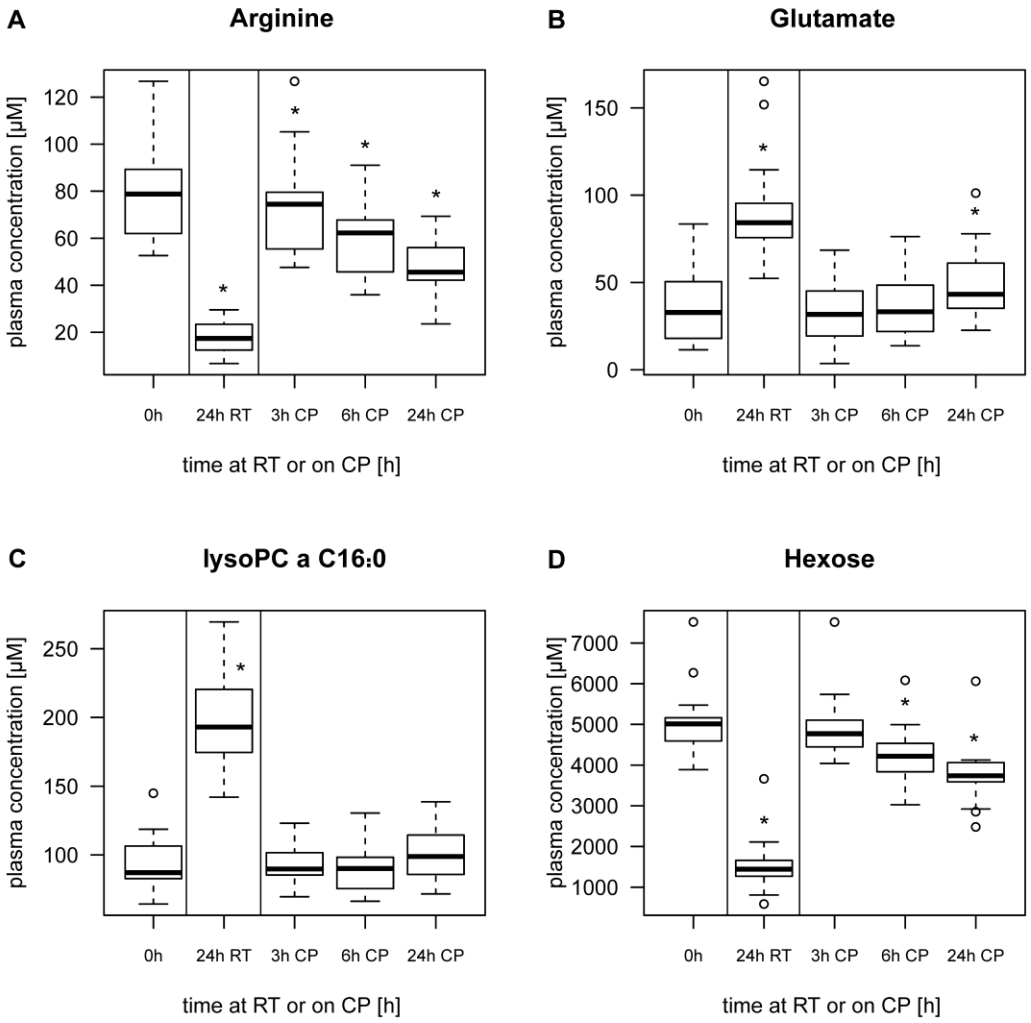

FIGURE 7. Stability of metabolites in plasma during simulated shipment.

Examples of (A), (D) decreasing and (B), (C) increasing metabolite concentration of plasma samples at RT and on CP. Vertical lines in boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).

The compound class of amino acids was the least stable metabolite class. In the compound class of biogenic amines, 77% of the investigated metabolite concentrations were stable for at least 24h on CP, whereas 46% remained stable at RT. 89% of the metabolite levels in the group of acylcarnitines maintained stable on CP for at least 24h and 70% for at least

24h at RT. Regarding the group of glycerophospholipids (including lysoPCs, PC aa's and PC ae's), all metabolites had stable concentrations for at least 24h on CP. Concentrations in two out of ten lysoPCs (lysoPC a C28:0 and lysoPC a C28:1) were even stable at RT for at least 24h. Concentrations in 32 of 36 PC aa's and all PC ae concentrations remained stable at RT. The compound class of sphingolipids was the most stable one, as all analyzed sphingolipid concentrations were stable on CP and at RT for at least 24h.

3.3.2 Effect of simulated shipment on metabolite ratios in plasma

In addition to 158 absolute metabolite concentrations, all possible pairs of metabolite ratios were calculated for plasma baseline samples and for plasma samples stored for 24h at RT. Those ratios might display enzymatic activity [131] and are therefore highly interesting for clinical studies. Within 24h at RT 74 metabolite ratios significantly changed (SUPPLEMENTARY TABLE 5). Each significantly changed ratio included at least one PC. Fifteen significantly altered ratios of PC aa's, seven ratios of PC ae's and 29 ratios of PC aa's to PC ae's were detected. In addition, ratios of Val/PC, Gly/PC, C2/PC and the ratios of three Sphingolipids with PCs were significantly elevated after 24h at RT compared with plasma baseline samples ($p = 3.81E-06$).

3.3.3 Effect of simulated shipment on serum metabolites

Comparing metabolite concentrations in plasma with concentrations in serum samples, the direction of the effect (i.e., significantly increased or decreased metabolite concentrations) in plasma samples during shipment simulation was similar in serum samples on CP over time (SUPPLEMENTARY FIGURES 11-15). In serum, a smaller number of metabolites had stable concentrations for 24h on CP as compared with plasma. In total, 140 of 158 metabolites in serum samples had stable concentrations on CP for at least 24h. In serum

18 metabolites showed significant changes in concentration during storage on CP within the first 24h (TABLE 4).

TABLE 4. Impact of simulated shipment on metabolite concentrations in serum samples.

Metabolites that showed significant changes in serum concentration on cool packs (CP) for 3, 6 or 24h compared with baseline (0h) (Friedman test, $p < 0.01$) and acceptable delay time for each metabolite during transportation (Wilcoxon signed rank test, $p < 0.01$).

Metabolite	p-value (Friedman)	Acceptable delay time on CP
C18:1	1.92E-04	3h
C18:2	2.52E-03	6h
Arg	1.52E-04	3h
Asn	1.49E-06	6h
Asp	6.08E-11	3h
Glu	1.20E-10	0h
Gly	4.79E-05	6h
Leu	1.01E-03	6h
Lys	8.49E-04	6h
Orn	1.18E-09	3h
Phe	2.69E-05	6h
Ser	9.04E-07	6h
Thr	1.86E-03	6h
Putrescine*	1.21E-06	0h
Serotonin*	2.67E-03	3h
Spermidine*	4.17E-04	0h
Taurine	1.00E-07	3h
Hexose	6.13E-07	3h

*Metabolites with CV across all plates ≥ 25% in reference samples

In 13 metabolites the concentration significantly increased over time (SUPPLEMENTARY FIGURES 11-15), whereas five metabolite concentrations significantly decreased (Arg, putrescine*, serotonin*, spermidine* and hexose). FIGURE 8 shows examples of altered metabolite concentrations in serum. In concordance with plasma samples, the group of amino acids was the least stable of all serum metabolites. 69% of the metabolites in the group of biogenic amines and 93% of the metabolites in the group of acylcarnitines showed

stable concentrations for at least 24h on CP. In the compound class of glycerophospholipids and sphingolipids, all metabolite concentrations remained stable on CP for at least 24h.

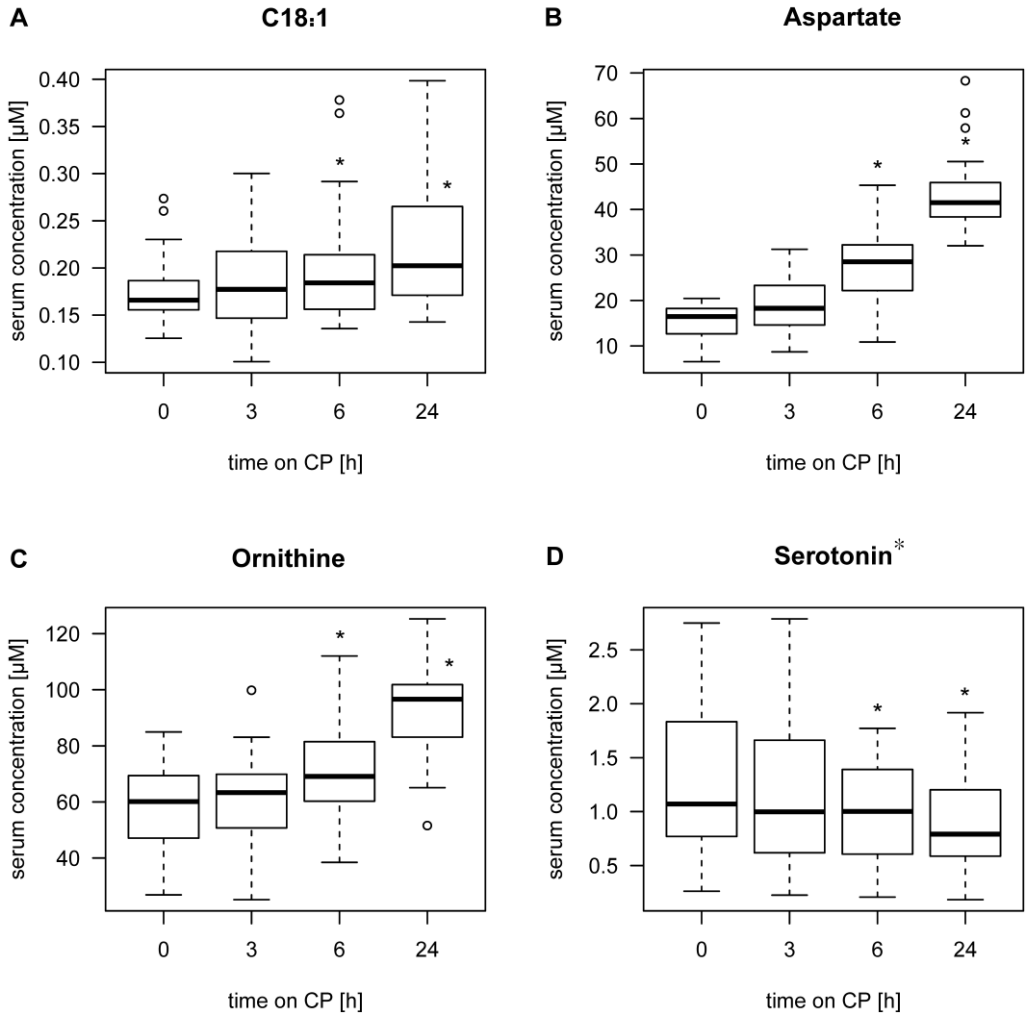

FIGURE 8. Stability of metabolites in serum during simulated shipment.

Examples of (A)-(C) increasing and (D) decreasing metabolite concentration during transportation simulation of serum samples on CP. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).

3.3.4 Effect size

The minimal effect size that could be detected for each metabolite with a given power of 0.8 and a significance level of 0.01 was calculated in plasma for the observed variance in metabolite concentration changes (SUPPLEMENTARY TABLE 2). This effect size shows the minimum absolute difference in metabolite concentrations between baseline concentrations and shipment simulation for 24h on CP or at RT that is detectable for a sample size of 20. For 105 of 158 metabolites the minimal detectable effect size is higher for the comparison of baseline samples with samples that were stored for 24h at RT compared to the minimal detectable effect size of the comparison of baseline samples with samples stored for 24h on CP. The present study was powered to significantly detect larger effects than those displayed in SUPPLEMENTARY TABLE 2. The effect size and the variance in metabolite concentrations after storage under different conditions are informative for the calculation of the sample size during the planning of future studies. Especially if shipment is performed at RT, a higher number of samples is necessary to significantly detect changes in metabolite concentrations that are not the cause of pre-analytical variation.

3.4 EFFECT OF FREEZE THAW-CYCLES ON SERUM METABOLITES

As repeated freezing and thawing of samples is often inevitable in multicenter clinical trials, the effect of one and two freeze-thaw cycles on metabolite levels was investigated in serum samples from 15 healthy participants.

After two freeze-thaw cycles, most metabolite concentrations remained stable in serum samples. Except for Met-SO, where concentrations decreased significantly, all 157 investigated metabolites of this panel showed stable concentrations after one freeze-thaw cycle. Eleven out of 158 metabolites revealed significantly decreased concentrations after

two freeze-thaw cycles, including three amino acids (Ile, Trp and Val), acetylornithine, the medium-chain acylcarnitine C10:1, five PCs (PC aa C32:2, PC ae C36:2, PC ae C36:5, PC ae C40:1 and PC ae C42:0) and SM C16:0 (TABLE 5).

TABLE 5. Effect of freeze-thaw cycles on serum metabolite concentrations.

Changes in serum metabolite concentrations after one and two freeze-thaw cycles, respectively, each tested against serum gel control (Wilcoxon signed rank, significance level $p < 0.01$).

		Effect of one thawing cycle	Effect of two thawing cycles
Metabolite	**p-value (Friedman)**	**p-value (Wilcoxon)**	**p-value (Wilcoxon)**
C10:1	6.07E-03	4.41E-02	9.44E-03
Ile	5.28E-03	7.59E-02	1.99E-03
Trp	6.42E-03	6.37E-02	2.33E-03
Val	9.43E-03	1.06E-02	2.33E-03
Acetylornithine	4.42E-03	2.40E-02	7.08E-04
Met-SO	6.79E-03	2.33E-03	1.72E-02
PC aa C32:2	3.88E-03	4.00E-02	1.21E-03
PC ae C36:2	3.67E-03	8.97E-02	6.39E-03
PC ae C36:5	5.14E-03	2.31E-01	5.58E-03
PC ae C40:1	2.79E-04	1.65E-01	2.33E-03
PC ae C42:0	2.93E-03	1.36E-02	6.39E-03
SM C16:0	8.25E-03	1.14E-01	9.44E-03

3.5 IMPACT OF LONG-TERM STORAGE ON METABOLITES

Plasma samples of a Danish study cohort were stored for over 18 years (baseline samples) and 8 years (follow-up samples) at -20°C. Here, a possible procedure on how to decide whether results after targeted metabolomics measurements are reasonable is presented by using this Danish study cohort as an example.

3.5.1 Technical variance

For a randomly selected subset of 13 healthy male participants, samples from the baseline and the follow-up visit were used for a targeted metabolomics measurement. To distinguish between technical variance derived from the measurement and biological variance derived from the samples, each specimen was measured in technical triplicates. For the baseline samples technical CVs with a value over 0.25 were detected in 1.6% and for the follow-up samples in 3.7% of the analyzed metabolites.

3.5.2 Comparison of metabolite levels with reference range

Mean values of technical replicates of 116 metabolites were compared with a reference range [28]. Metabolites with a significantly (McNemar test, $p < 0.01$) higher proportion of cases in which (c): the baseline sample of an individual was out of range and the follow-up sample was within the reference range, compared with (b): the baseline sample of an individual was within the reference range and the follow-up sample was out of the reference range, are displayed in TABLE 6.

In 21% of the investigated metabolites, baseline samples were significantly more often ‘out of reference range’ while follow-up samples from the same individuals were within range. The compound class of PCs was most affected by long-term storage at -20°C. 28% of the investigated PCs were significantly more often defined as ‘out of reference range’ in the baseline samples compared with the follow-up samples. Furthermore, metabolites of the groups of amino acids and acylcarnitines showed significantly stronger aberrations from the reference range in the baseline samples compared with the follow-up samples. Metabolite levels within the groups of lysoPCs, sphingolipids and hexose did not differ significantly between the baseline and the follow-up samples.

TABLE 6. Impact of long-term storage on metabolite levels.

(c): Number of metabolites with significantly higher proportions of baseline samples 'out of reference range' while follow-up samples are within reference range, compared with (b): number of metabolites with significantly higher proportions of baseline samples within reference range while follow-up samples are 'out of reference range' within the same individuals (McNemar test, $p < 0.01$).

Metabolite	p-value	c	b
C2	9.37E-03	11	1
C3	5.55E-03	12	1
Gln	9.37E-03	11	1
His	5.55E-03	12	1
Met	9.37E-03	11	1
PC aa C34:2	4.43E-03	10	0
PC aa C36:0	9.37E-03	11	1
PC aa C36:4	9.37E-03	11	1
PC aa C38:0	9.37E-03	11	1
PC aa C38:4	9.37E-03	11	1
PC aa C38:5	5.55E-03	12	1
PC aa C38:6	9.37E-03	11	1
PC aa C40:2	5.55E-03	12	1
PC aa C40:3	5.55E-03	12	1
PC aa C42:1	9.37E-03	11	1
PC aa C42:2	9.37E-03	11	1
PC ae C30:2	5.55E-03	12	1
PC ae C36:5	5.55E-03	12	1
PC ae C38:3	5.55E-03	12	1
PC ae C40:2	5.55E-03	12	1
PC ae C40:3	9.37E-03	11	1
PC ae C42:1	5.55E-03	12	1
PC ae C42:2	5.55E-03	12	1
PC ae C44:3	5.55E-03	12	1

3.6 METABOLIC CHANGES INDUCED BY METFORMIN

As an example of a multicenter study, targeted metabolomics measurements were performed to detect changes in serum samples of previously untreated individuals with

T2DM, that were provoked by a single dose of metformin followed by a short period of metformin monotherapy (4–6 weeks).

3.6.1 Impact of the first dose of metformin on metabolism

In the discovery study (*n* = 30), a significant change in Cit concentration between time point A (before first dose of metformin) and time point B (10–16h after the first dose) was observed after correction for multiple testing. Five additional metabolite traits showed nominally significant ($p < 0.05$) changes. These six metabolite traits were further investigated in the replication study (*n* = 164). For five of these six metabolite traits, results were replicated by showing significant changes between time points A and B in the replication study (TABLE 7). Cit concentration decreased significantly after the first dose of metformin (FIGURE 9).

TABLE 7. Changes in metabolite traits after the first dose of metformin.

Beta estimate and p-values in the linear mixed effects regression model for significant changes in metabolite concentrations between time point A (baseline) and time point B (after the first dose of metformin) in the discovery and replication studies ($p < 0.05$ for discovery and $p < 2.78E-03$ for replication).

	Discovery study		Replication study	
Metabolite	**Beta estimate**	**p-value**	**Beta estimate**	**p-value**
Cit	-8.02	4.68E-05	-4.14	1.83E-10
Cit/Orn	-0.09	5.40E-04	-0.04	3.21E-05
Cit/Arg	-0.06	4.72E-03	-0.06	2.46E-05
C8	0.02	3.01E-02	0.02	4.16E-04
C10:1	0.02	5.37E-03	0.01	2.65E-04

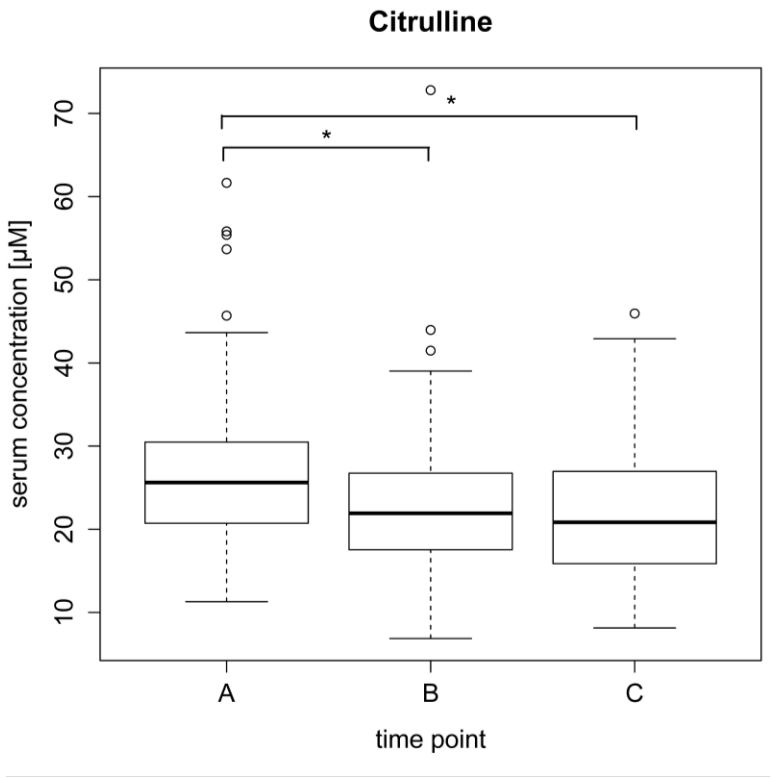

FIGURE 9. Concentrations of Cit at baseline (A), after the first dose of metformin (B), and after 4–6 weeks of metformin monotherapy (C) in the replication study.

Asterisks in boxplots indicate significant differences in concentration compared with time point A. (Linear mixed effects model, p < 2.78E 03).

The ratio of Cit to Orn and the ratio of Cit to Arg significantly decreased, whereas concentrations of two acylcarnitines, i. e., octanoylcarnitine (C8) and decenoylcarnitine (C10:1) (FIGURE 10), significantly increased after the first dose of metformin (see TABLE 7).

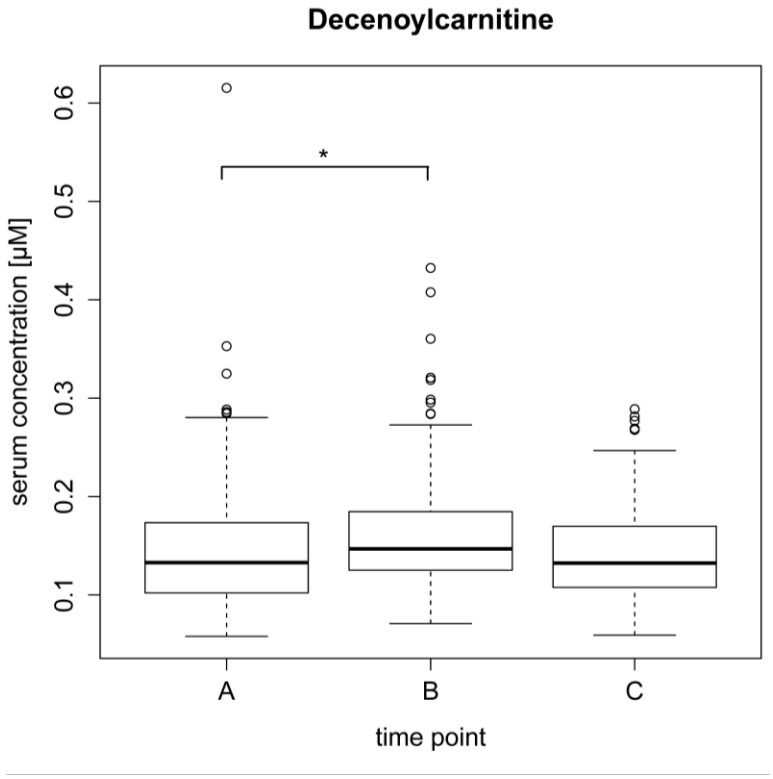

FIGURE 10. Concentrations of C10:1 at baseline (A), after the first dose of metformin (B), and after 4–6 weeks of metformin monotherapy (C) in the replication study.

Asterisk in boxplots indicate a significant difference in concentration compared with time point A. (Linear mixed effects model, p < 2.78E 03).

Differences in the metabolomic response between male and female subjects to the first metformin dose were investigated in a sex-stratified analysis. Here, six metabolite traits that showed nominally significant differences in the discovery study could not be replicated.

3.6.2 Impact of short-term metformin intake on metabolism

After 4–6 weeks of metformin monotherapy (time point C) a third fasting serum sample was collected from a subgroup of participants. In the discovery study, 15 metabolite traits showed nominally significant changes between time point A and time point C. Four out of these 15 were replicated (TABLE 8).

Cit concentration decreased significantly from time point A to time point C, but the concentration at time point C did not differ significantly from the concentration at time point B (discovery: p = 0.75; replication: p = 0.06). In addition, the ratio of Cit to Orn decreased significantly from time point A to time point C. Furthermore, the ratio of hydroxylated to non-hydroxylated sphingomyelins (total SM-OH/total SM-non-OH) and ratio of the sum of BCAA to the sum of AAA significantly increased between time points A and C. Except for Cit and Cit/Orn, changes were not significant between time points A and B.

TABLE 8. Changes in metabolite traits after short-term metformin therapy.

Beta estimates and p-values in the linear mixed effects regression model for significant changes in metabolite levels between time point A (baseline) and time point C (after 4–6 weeks of metformin therapy) in the discovery and replication studies.

	Discovery study		Replication study	
Metabolite	**Beta estimate**	**p-value**	**Beta estimate**	**p-value**
Cit	-8.70	1.57E-04	-4.95	1.61E-11
Cit/Orn	-0.08	1.28E-02	-0.05	4.77E-05
Total SM-OH/Total SM-non OH	0.01	7.32E-03	0.00	1.42E-03
BCAA/AAA	0.13	3.87E-02	0.14	3.60E-07

A nominally significant sex-specific effect was detected for Methylglutarylcarnitine (C5-M-DC) (p = 0.04) in the discovery study. This finding was not replicated. All metformin-induced metabolic changes observed in the analyses were independent of sex, baseline HbA_{1c}, and age of patient.

4. DISCUSSION

4.1 RELIABILITY OF PLASMA AND SERUM METABOLITES

The analysis of three fasting blood samples within a 14 day time period in healthy humans showed that most of the 158 analyzed metabolite levels remained stable within the same individual. Plasma and serum metabolites showed good reliability within individuals. An ICC above 0.50 was defined as good reliability [42]. In the present study, more than 75% of metabolites in plasma and serum samples fulfilled this criterion. Compared with a population-based approach, ICC values in this study were artificially low due to the homogeneous study cohort. Reliability of metabolites was slightly higher in serum than in plasma.

The observed good reliability of amino acids was consistent with the results of Floegel et al. [42], where they investigated serum metabolite concentrations over a 4-month period using a targeted metabolomics approach. However, reliability for six additional amino acids was shown in the present study. Midttun et al. showed excellent reliability (ICC ≥ 0.75) for Gly and Ser and good to fair reliability (ICC = 0.74–0.40) for Arg and Trp, whereas reliability for Met was poor (ICC = 0.39–0.15) in human plasma samples [24].

In the present study creatinine was found to have the lowest WCV and highest reliability in plasma. This is in line with Midttun et al. [24]. In muscle cells, the ring closing of creatine forms creatinine in a chemical equilibrium [132]. As the quantity of creatine per unit of skeletal muscle and the reaction to creatinine is constant [133], the produced amount of creatinine is dependent on the muscle mass. Concentration of creatinine is therefore constant and within-person variability is relatively low in healthy people, as long as the muscle mass remains constant. Furthermore, good to fair reliability of ADMA, kynurenine, and symmetric dimethylarginine (SDMA) as well as poor reliability of Met-SO were reported recently [24].

In the present study, reliability was lowest for acylcarnitines, as hydroxyacylcarnitines showed low reliability due to low concentrations. Low reliability of long chain (>C10) and unsaturated acylcarnitines was induced either by low concentration or by higher within-person variability compared with between-person variability. The classification of the reliability of acylcarnitines was concordant with that of Floegel et al. [42]. They found that most of the short- and medium-chain saturated acylcarnitines showed good reliability, whereas reliability of hydroxyacylcarnitines and monounsaturated acylcarnitines was low [42]. The good reliability of phospholipid measurements in plasma samples [134] and serum samples [42] is in line with the results of the present study. In addition, good reliability was reported for the compound class of sphingolipids [42] which was also observed in the present study. Widjaja et al. showed that a single measurement of glucose is sufficient in plasma samples [25] and reliability of hexose in serum samples was defined as excellent by Floegel et al. [42]. Here, reliability of the sum of hexoses was good.

In serum samples 101 metabolite concentrations were significantly higher compared with plasma. Similar results were observed for 86 of these metabolites in Yu et al. [135]. Two of the 101 metabolites (C10:2 and PC aa C40:2) were excluded by them and twelve of these 101 metabolites were not part of their metabolite panel. In the present study, SM (OH) C24:1 showed significantly higher concentrations in serum compared with plasma. This finding is not in line with Yu et al. [135]. Ladenson et al. found a 5% lower glucose concentration in plasma compared with serum [136]. Although in the present study concentration of the sum of hexoses was lower in plasma samples, the difference between plasma and serum levels was not significant.

In contrast to other studies [28,30,31], no effect of sex and last meal composition before overnight fasting was found. This could be explained by the small sample size and the unbalanced ratio of men to women in the present study.

4.2 EFFECT OF SERUM GEL-BARRIER TUBES ON METABOLITES

The impact of tubes with a gel separator on metabolite concentrations was investigated by comparing samples drawn into serum W tubes and serum gel-barrier tubes. Only Met-SO showed significant changes of concentration in gel-barrier tubes compared with serum W tubes.

This finding might indicate a possible interaction of the gel with the investigated specimen. Therefore, the use of serum gel-barrier tubes is not recommended, if the concentration of Met-SO is of interest. However, the use of serum gel-barrier tubes should be preferred for all other analyzed metabolites in multicenter settings due to the advantages in sample handling.

Cuhadar et al. detected no effect for creatinine and glucose levels in samples drawn in serum gel-barrier tubes compared with samples from serum W tubes stored at 4°C and at RT for up to three days [54], whereas in the present study significantly decreased concentrations of the sum of hexoses were observed after 6h on CP. Lima-Oliveira et al. showed changes in concentrations of creatinine but not glucose, by comparing samples drawn in serum gel-barrier tubes of different manufacturers [64]. It has been reported previously that the gel inside the tube interacts with several drugs, which leads to decreased concentrations of the investigated component in the serum [56,57,63]. Especially, hydrophobic drugs tend to adsorb to the hydrophobic gel matrix [60]. Furthermore, a possible release of substances out of the separator gel, which might interfere with downstream analyses, was reported in several cases [53,62].

4.3 EFFECT OF SIMULATED SHIPMENT ON METABOLITES

The majority of the analyzed metabolites had stable concentrations for at least 24h on CP (both plasma and serum) and at RT (plasma only). In plasma and serum samples, the

compound class of amino acids was most unstable during simulated transportation. No significant changes in concentration during transportation for 24h on CP and at RT were detected for PC ae's and sphingolipids. Metabolite stability in plasma and serum showed good coherence. In plasma, more metabolites maintained stable on CP compared with metabolites in serum (145 stable metabolites on CP in plasma vs. 140 in serum).

Except for Arg and Met, all investigated amino acid levels had an increased trend over time, which might show the degradation of proteins, especially at RT. LC-MS measurement of 18 amino acids in porcine cerebrospinal fluid, stored at RT for up to 2h, showed increased concentrations in ten amino acids (Ala, Asn, Gly, Glu, His, Ile, Phe, Ser, Thr, Tyr) [71], which is in line with observations in the present study, except that Gly concentration was stable in the present experiments. Yang et al. showed stability of nine amino acids in rat plasma for up to 24h at 37°C [72]. Regarding plasma samples, only Trp and Val stability is in accordance with present results. In a study from Midttun et al., Arg levels were shown to decrease significantly in plasma samples after 24h and after 48h on ice packs, whereas Gly, Met, Ser, and Trp maintained stable [24]. Furthermore, in human plasma samples the concentrations of Gln, Glu, Leu, Orn, and Pro significantly changed at different settings compared with baseline, whereas levels of Met were stable [73].

Ono et al. reported stable creatinine concentrations in serum samples after a delay of up to 48h at 4, 24 or 30°C [74], whereas Cuhadar et al. detected stable creatinine levels after a delay for at least three days at 4°C and at RT [54]. Levels of ADMA, creatinine, kyurenine, Met-SO, and SDMA remained stable in unprocessed plasma samples for up to 48h on ice packs in a study of Midttun et al [24]. Further, stable concentrations of ADMA, creatinine, Met-SO, and SDMA were observed by Hustad et al. in human plasma samples during storage at RT for up to eight days [75].

As most acylcarnitines showed stable concentrations over time, the anabolic and catabolic metabolism of acylcarnitines seems to be balanced during the delay of sample processing. Within the group of acylcarnitines, Yang et al. showed decreasing concentrations of C8,

C18:1 and C18:2 and stable concentrations of carnitine and eight other acylcarnitines (C2, C3, C4, C5, C6, C14, C16, C16:1) at 37°C after 24h [72]. The stability of carnitine, C2, C3, C4 and C5 for 24h at 37°C is in line with the present results (24h at RT), whereas C18:1 and C18:2 showed an opposing trend. Stability of C14 and C16 was not confirmed in the present study. In contrast to Yang et al., the results presented here showed stability of C8 for 24h at RT. The reason for these partly contradictory results could be the small size of three subjects in Yang et al. and the different origin of specimen (rat in Yang et al.).

The decreasing levels of four PC aa's and increasing levels of eight lysoPCs during storage at RT could be explained by the hydrolysis of PC to yield a lysophospholipid and free fatty acid, catalyzed by a phospholipase [137]. This result might reflect remaining activity of phospholipase in non-centrifuged plasma samples at RT, but not at 4°C. Increasing lysoPC levels at RT were also observed in Yang et al. [72]. In the present study ratios of PC aa's and PC ae's significantly changed in plasma samples after 24h at RT. This finding might reflect activity of alkylglycerone phosphate synthase (alkyl-DHAP) at RT as this enzyme catalyzes the oxidation of long-chain alcohols to long-chain acids [138].

Sphingomyelins, which were found to play an important role in β-cell function [139], were the most stable compound class both in plasma and serum samples in the present study. This finding indicates low enzymatic activity of sphingomyelin degrading and synthesizing enzymes at 4°C and at RT.

Decreasing hexose concentrations on CP over time and especially at RT are consistent with the literature [50,54,73,140] and could be explained by activity of glycolytic enzymes.

The higher stability of plasma metabolites compared with serum could be explained by the use of the metal-binding EDTA in the plasma tubes. EDTA deactivates metal-dependent enzymes as metal ions in the plasma are chelated [141]. Compared with other matrices stored at RT, biomarkers were found to be most stable in EDTA plasma [75].

4.4 EFFECT OF FREEZE THAW-CYCLES ON SERUM METABOLITES

Except for Met-SO, all 158 investigated metabolites had stable concentrations after one freeze-thaw cycle. After two freeze-thaw cycles, only eleven metabolites showed significantly decreased concentrations.

The considerable influence of one freeze-thaw cycle on Met-SO might arise from the interaction of this metabolite with the gel matrix in the tube. Repeated freezing and thawing could impair this effect.

Investigations using targeted and non-targeted metabolomics were performed regarding the stability of several plasma and serum metabolites in repeated freeze-thawing experiments [37,80,84-86]. They likewise showed only minor changes in metabolite concentrations due to multiple thawing and freezing.

In accordance with the results of the present study, creatinine was found to have stable concentrations in serum samples for up to ten freeze-thaw cycles [84]. Zivkovic et al. reported significantly altered concentrations of lysoPC a C16:0 in serum samples after two freeze-thaw cycles, whereas levels of lysoPC a C18:0 and PC aa C24:0 were not affected [86].

In rat serum, significantly higher concentrations of glucose were found after two freeze-thaw cycles by Kale et al. [85], but not after three freeze-thaw cycles. The author deduced that increased glucose levels could not be imputed to the effect of repeated freezing and thawing. Another study reported no changes in glucose concentration for up to 10 freeze-thaw cycles in human serum samples [84].

4.5 IMPACT OF LONG-TERM STORAGE ON METABOLITES

From a subgroup of 13 healthy male subjects of a Danish study cohort, plasma samples from a baseline- and a follow-up visit stored at -20°C for over 18 years and 8 years, respectively, were selected for targeted metabolomics measurement. Metabolite levels of the baseline and the follow-up samples were measured in technical replicates and afterwards compared to a reference range [28]. Results show low technical variance in this sample set. Amino acids, acylcarnitines and PCs reveal a significantly higher proportion of cases where the baseline samples are out of the reference range while the follow-up samples within the same individual are within the reference range, compared with the observation that the baseline samples are within the reference range while the corresponding follow-up samples are out of the reference range.

These findings indicate that, regarding targeted metabolomics, samples of the baseline visit are of lower quality compared with samples of the follow-up visit and will therefore not be sound for the metabolomics platform. Measured differences for this study cohort might be due to inappropriate storage conditions rather than real differences in metabolite concentrations. Previous studies dealt with the question of the stability of different analytes during long-term storage [72,75,88,142-144]. The concentration of Met-SO in human serum samples significantly increased during long-term storage for up to 29 years at -25°C, whereas concentrations of Met decreased [75]. Storage at -20°C for one month significantly elevated the concentrations of Pro and glucose in human plasma samples [142]. Significant differences in metabolite concentration between LC/MS-MS measurements of human plasma samples stored for two months at -80°C and repeated measurements after storage for five years at -80°C were detected by Yang et al. [72]. Changes in concentration affected lysoPCs, lysophosphoethanolamines, certain acylcarnitines (C8-OH, C18:1, C18:2, and C20:3), serotonin and hypoxanthine. However, no associations between untargeted

metabolite profiles measured in plasma samples at baseline and after 13 to 17 years of storage at -80°C were detected by Hebels et al. [88].

These studies compared measurements of samples at baseline with data from the same samples obtained after long-term storage. In order to perform their approach, information about the concentration of the investigated analyte at baseline was required. However, in many cohort studies, sample collection ranges over several years while measurement is performed only after completion of sample collection. Furthermore, metabolomics is a relatively new field of research and the characterization of the human metabolome is not finished [145]. Thus, newly identified metabolites for which the long-term stability during storage has not yet been determined may be able to be measured in the future [144]. For this reason, their approach is not appropriate, if the analysis of metabolites is of interest. The advantage of the method presented here is that no information about analyte concentrations right after sample collection is required to evaluate the sample quality and suitability for targeted metabolomics. Furthermore, this method gives a first hint on possible changes of metabolite concentrations caused by inappropriate sample storage. It can be performed with any study cohort with plasma samples of healthy male subjects, especially if the analysis of amino acids, acylcarnitines, glycerophospholipids, sphingolipids and hexose is of interest.

4.6 METABOLIC CHANGES INDUCED BY METFORMIN

4.6.1 Impact of the first dose of metformin on metabolism

A significant decrease in Cit levels as well as in the Cit/Orn and Cit/Arg ratios was observed in response to the first dose of metformin in newly diagnosed T2DM patients. This shows an immediate impact of the drug on the urea cycle. The non-proteinogenic amino acid Cit is formed in the urea cycle by the condensation of Orn and carbamoylphosphate, catalyzed by

Orn transcarbamoylase (OTC, EC 2.1.3.3) [146]. A significant decrease in the Cit/Orn ratio reflects reduced activity of this enzyme [146]. The Cit/Arg ratio is an indicator of the activity of nitric oxide synthase (NOS, EC 1.14.13.39) [147]. The reduced Cit/Arg ratio after the first metformin dose may therefore reflect an inhibitory effect of metformin on the activity of this enzyme within the urea cycle (FIGURE 11).

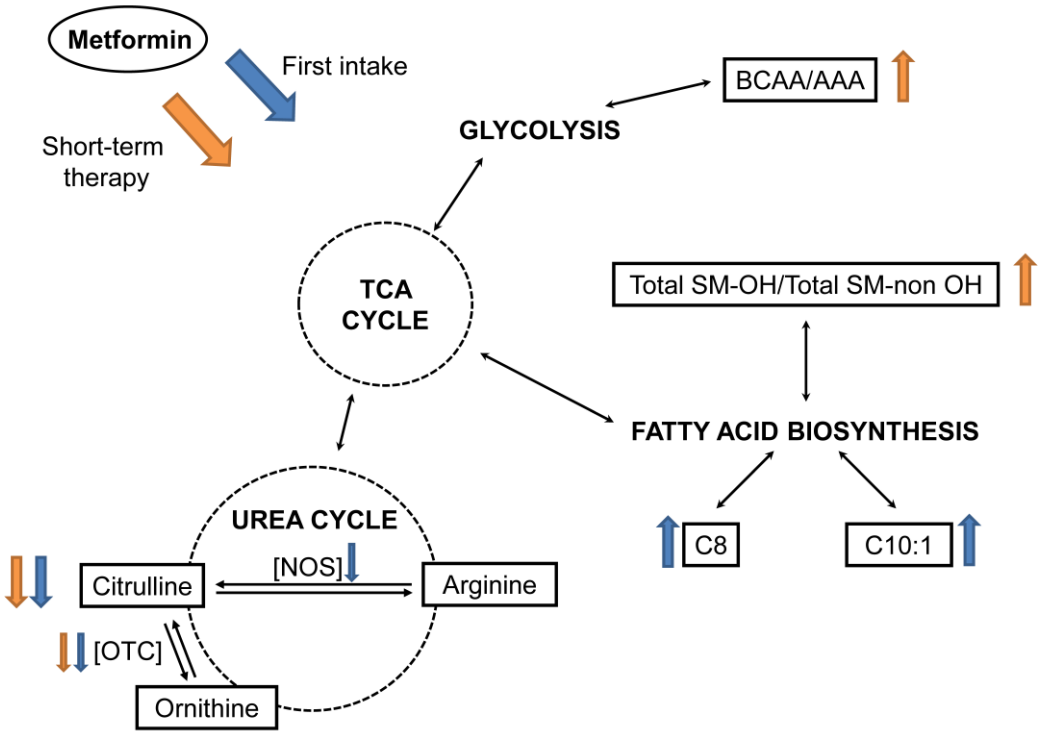

FIGURE 11. Schematic of changes in metabolite traits after first intake (blue) and after short-term metformin therapy (orange) in patients with T2DM.

Glucogenic amino acids which are processed in the urea cycle are, in part, used as substrates in gluconeogenesis. Metformin inhibits gluconeogenesis [148,149] and ATP production [119]. As gluconeogenesis is an energy consuming process, it is hypothesized that one mode of action of metformin is the partial inhibition of the urea cycle, which leads to feedback inhibition of gluconeogenesis. Increases in urea cycle-related metabolites, particularly Cit, correlate with obesity [150] and with an elevated consumption of amino acids for gluconeogenesis during the development of insulin resistance [98]. Therefore,

augmented hepatic urea cycle activity could be an early characteristic of hepatic insulin resistance [98]. This idea is supported by the finding that blood Cit levels are significantly raised in individuals with untreated T2DM compared with healthy controls [151]. Furthermore, Shaham et al. reported decreased concentrations of urea cycle intermediates, including Cit, after oral glucose ingestion in healthy humans [152]. This likely reflects the down-regulation of gluconeogenesis following a meal and supports a close association between hepatic amino acid metabolism and gluconeogenesis. Taken together, these findings indicate that in insulin resistant individuals the activity of the hepatic urea cycle is coupled to gluconeogenesis and it is suggested that metformin, by its energy depleting effect, partially blocks this metabolic pathway, which in turn reduces hepatic glucose production.

As acylcarnitines are metabolic intermediates of fatty acid oxidation [153], the here observed increase in C8 and C10:1 after metformin intake might reflect a change in cellular fatty acid handling. However, the exact nature of this alteration remains to be determined. Acylcarnitines in the context of T2DM have been previously investigated [105,107,154], and long-chain acylcarnitines were shown to be associated with insulin resistance [155]. Mai et al. observed elevated serum levels of C2, C6, C8:1, C10:1, C14:1, C16-OH, and C4-OH (C3-DC) in T2DM patients compared with subjects with normal glucose tolerance, whereas concentrations of C8 did not differ [154]. Furthermore, significantly higher levels of C8 and C10:1 were reported in fasting plasma samples of obese T2DM patients compared with samples of lean subjects with normal glucose tolerance [106]. In another study, higher concentrations of C8 were detected in females with T2DM compared with non-diabetic females [105]. However, in that study, no information was provided on the medication of the participants.

4.6.2 Impact of short-term metformin intake on metabolism

A third blood sample was collected from a subgroup of participants 4–6 weeks after the start of metformin therapy. It is likely that the metabolic status had already changed at this time, as a result of the treatment. Therefore, changes in metabolite traits which were found in the third blood sample compared with the baseline sample may not only reflect metformin intake, but also improved insulin resistance and glucose metabolism [6].

The concentration of Cit and the Cit/Orn ratio remained significantly lower after 4–6 weeks of metformin monotherapy compared with baseline levels. These findings suggest decreased Cit concentrations after the first dose of metformin, and further treatment led to stabilization of Cit concentrations at a lower level than before the initiation of treatment. This indicates a persistent effect of metformin on the urea cycle.

In addition, the ratio of total SM-OH to total SM-non-OH and the ratio of BCAA to AAA significantly increased after 4–6 weeks of metformin monotherapy. The ratio of total SM-OH to total SM-non-OH may provide information on the activity of fatty acid 2-hydroxylase (FA2H), an enzyme which catalyzes the C-2 hydroxylation of fatty acid side chains in sphingolipids [156]. However, the direct hydroxylation of N-acyl chains of sphingolipids by FA2H has not yet been examined [156]. Interlipid association is assumed to be stabilized by 2-hydroxylation [157] and FA2H knock-out in adipocytes leads to impaired basal and insulin-stimulated glucose uptake, a decreased level of insulin receptor proteins, and inhibition of lipogenesis [158,159]. In the present study, the elevated ratio of total SM-OH to total SM-non-OH after 4–6 weeks of metformin treatment may indicate an increased activity of FA2H, either directly induced by metformin or by an overall lower level of insulin resistance.

An elevated BCAA/AAA ratio during metformin therapy has been reported previously [160]. In that same study, altered levels of BCAA and AAA were associated with incident T2DM [160]. A reduction in the insulin/glucagon ratio, which occurs in T2DM, may be

connected to an impaired balance between amino acid anabolism and catabolism [161]. Based on the findings of the present study, it is speculated that metformin contributes to the restoration of the disturbed balance of BCAA/AAA in T2DM.

To the best of knowledge, this is the first study which used a targeted metabolomics approach to analyze the intra-individual immediate and short-term effects of metformin on metabolism of humans with T2DM. Huo et al. [162] compared serum metabolites in T2DM patients after three months of metformin treatment with an untreated control cohort and found increased trimethylamine-N-oxide and 3-hydroxybutyrate, and decreased glucose, N-acetyl glycoprotein, lipoprotein, lactate, acetoacetate, as well as unsaturated lipids. In addition, they detected increased Trp and decreased lysoPCs (lysoPC a C16:0, lysoPC a C18:0, and lysoPC a C18:2) and Phe in the metformin-treated group [162]. Of these metabolites, Trp, lysoPCs, and Phe were also measured in the present study, but no significant differences between the concentrations before and after treatment were found. Possible reasons for this discrepancy are the longer duration of metformin treatment and the between-subject analysis in the study by Huo et al., which differed from the present within-subject approach. Another study of newly diagnosed T2DM patients compared serum samples before metformin treatment with samples taken 24 and 48 weeks after the initiation of treatment, respectively, and found decreased serum Glu after 24 and 48 weeks compared with pretreatment concentrations [101].

Previous studies have identified sex-dependent differences in the activity and side effects of several drugs [163,164]. Therefore, it was investigated whether metabolic changes induced by metformin differed between male and female study participants. No replicable significant differences were detected and it is suggested that metformin has comparable metabolic effects in both sexes. Sambol et al. [165] found no significant differences in metformin kinetics in males compared with females with and without T2DM.

4.7 STRENGTHS AND LIMITATIONS

The major strength of the present thesis is the broad metabolite panel for targeted metabolomics measurements. Regarding the reliability of plasma and serum metabolites, three samples per individual were analyzed in a well-established and validated targeted metabolomics approach. The calculated variance can help to estimate the appropriate sample size of future clinical trials. Furthermore, this was the first study which investigated the response of the first metformin intake on the human metabolome within the same individual.

This thesis has some limitations. No analytical duplicates were measured in the analysis of the reliability of metabolites. Therefore, it was not possible to further distinguish the total within-person variability into biological and analytical variability. However, regarding the between and within-plate CVs derived from repeated measurements of reference samples, analytical variance is assumed to be low. Furthermore, in the analyses of in vivo- and in vitro pre-analytical variations, only samples of healthy subjects were analyzed. Variability in non-healthy patients could vary from these results.

A general limitation of the presented studies is the sample size. A power calculation was performed for selected settings regarding effects of simulated shipment on metabolite concentrations and illustrated the minimum detectable effect size. The small sample size of the discovery study may have reduced the sensitivity for detecting changes in metabolite traits. However, the large number of replication samples makes the reported results very robust.

4.8 CONCLUSION

Based on the present data, the day to day fluctuation in healthy humans is low for most metabolites and therefore a single time point measurement sufficiently reflects the metabolic state of an individual. The optimal method of sample shipment in multicenter trials is to collect samples in serum in gel-barrier tubes, to centrifuge them at site and to ship the samples while frozen on ice, as at higher temperatures some amino acids and biogenic amines showed to be unstable in plasma and serum. The majority of metabolites were stable in non-centrifuged samples for up to 24h on CP, allowing for cost-saving transportation of samples.

Long-term storage of human plasma samples might lead to significant changes in the concentration of amino acids, acylcarnitines and PCs. Therefore, targeted metabolomics might not be suitable for samples stored at -20°C for several decades. It is recommended to perform the method presented here for the evaluation of sample quality and suitability before the analysis of whole sample sets, especially, when the quality of long-term stored samples is unknown, in order to save time and costs and to prevent from misinterpretation of the results.

Furthermore, as an application of targeted metabolomics a multicenter study using human serum samples was performed to gain new insights into the mode of action of metformin during the treatment of T2DM. The present study might show evidence of a partial inhibition of the urea cycle by metformin, which could be relevant for the glucose lowering effect of this drug. Altered beta oxidation, the activation of fatty acid 2-hydroxylase, and changes in amino acid metabolism are also potentially relevant effects of metformin. Overall, this thesis answers questions regarding the in vivo- and in vitro pre-analytical stability of metabolites and helps to incorporate targeted metabolomics into multicenter clinical trials. Furthermore, new aspects of the mode of action of the most important drug in T2DM treatment on the human metabolome were revealed.

4.9 OUTLOOK

Results obtained in the present thesis can help to judge whether single measurements of a metabolite are sufficient to describe differences between groups of human subjects. They can also help to interpret effects of a study medication or another intervention in a clinical trial. Furthermore, this thesis provides recommendations for pre-analytical sample handling of future clinical trials. Further studies might include samples obtained from patients with certain diseases. Depending on the metabolite panel, the choice of the right collection tube and the maximum number of freeze-thaw cycles is facilitated by the results presented here. Metabolites which are both reliable and stable under certain conditions were identified. This allows for the planning of clinical trials that include the shipment of non-processed plasma or serum at RT, which can be a simple and cost-effective alternative for selected questions. The presented method of detecting long-term storage variation of metabolite concentrations might help to evaluate the suitability of particular samples for targeted metabolomics. The usage of this method could be extended by additional reference ranges which are appropriate for specific questions.

Furthermore, the present findings provide a new starting point for further investigations on changes of metabolite concentrations induced by metformin. Especially, the analysis of altered metabolite levels within in the citric acid cycle, enzyme activities within the urea cycle, the levels of carbamoyl phosphate, nitric oxide, and urea might be of interest. The present results might also support the development of improved metformin-like therapeutics.

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APPENDIX

SUPPLEMENTARY TABLE 1. List of all investigated metabolite traits.

[1)] did not pass quality control in Munich study cohort (Reliability of plasma and serum metabolites, effect of serum gel-barrier tubes, simulated shipment, and freeze-thaw cycles on metabolites); [2)] in Danish study cohort; [3)] in participants with T2DM (metabolic changes induced by metformin).

Acylcarnitines	PC ae's	PC aa's	predefined sums and ratios
C0	PC ae C30:0	PC aa C24:0[2)]	AAA
C10	PC ae C30:1	PC aa C26:0[1) 2) 3)]	ADMA/Arg
C10:1	PC ae C30:2[1) 3)]	PC aa C28:1	BCAA
C10:2	PC ae C32:1	PC aa C30:0	C2/C0
C12[2)]	PC ae C32:2	PC aa C30:2[1) 2)]	Cit/Arg
C12-DC[1) 2) 3)]	PC ae C34:0	PC aa C32:0	Cit/Orn
C12:1[1) 2) 3)]	PC ae C34:1	PC aa C32:1	CPT-I ratio
C14[2)]	PC ae C34:2	PC aa C32:2	Essential AA
C14:1	PC ae C34:3	PC aa C32:3	BCAA/AAA
C14:1-OH[2)]	PC ae C36:0	PC aa C34:1	Glucogenic AA
C14:2[2)]	PC ae C36:1	PC aa C34:2	Kynurenine/Trp
C14:2-OH[2) 3)]	PC ae C36:2	PC aa C34:3	Met-SO/Met
C16	PC ae C36:3	PC aa C34:4	MUFA (PC)
C16-OH[1) 2) 3)]	PC ae C36:4	PC aa C36:0	MUFA (PC)/SFA (PC)
C16:1[1)]	PC ae C36:5	PC aa C36:1	Non-essential AA
C16:1-OH[1) 2) 3)]	PC ae C38:0	PC aa C36:2	Orn/Arg
C16:2[2)]	PC ae C38:1[3)]	PC aa C36:3	PUFA (PC)
C16:2-OH[1) 2) 3)]	PC ae C38:2	PC aa C36:4	PUFA (PC)/MUFA (PC)
C18[2)]	PC ae C38:3	PC aa C36:5	PUFA (PC)/SFA (PC)
C18:1	PC ae C38:4	PC aa C36:6	Putrescine/Orn
C18:1-OH[1) 2) 3)]	PC ae C38:5	PC aa C38:0	Serotonin/Trp
C18:2[2)]	PC ae C38:6	PC aa C38:1	SFA (PC)
C2	PC ae C40:1	PC aa C38:3	Spermidine/Putrescine
C3	PC ae C40:2	PC aa C38:4	Spermine/Spermidine
C3-DC (C4-OH)[1) 2)]	PC ae C40:3	PC aa C38:5	Total (PC+SM)
C3-OH[2) 3)]	PC ae C40:4	PC aa C38:6	Total AA
C3:1[1) 2) 3)]	PC ae C40:5	PC aa C40:1	Total AC/C0
C4	PC ae C40:6	PC aa C40:2	Total AC-DC/Total AC
C4:1[2)]	PC ae C42:0	PC aa C40:3	Total AC-OH/Total AC
C5	PC ae C42:1	PC aa C40:4	Totoal lysoPC
C5-DC (C6-OH)[1) 2) 3)]	PC ae C42:2	PC aa C40:5	Totoal lysoPC/Total PC
C5-M-DC[1) 2) 3)]	PC ae C42:3	PC aa C40:6	Total PC
C5-OH (C3-DC-M)[1) 2) 3)]	PC ae C42:4	PC aa C42:0	Total PC aa
C5:1[2)]	PC ae C42:5	PC aa C42:1	Total PC ae

C5:1-DC[2)]	PC ae C44:3	PC aa C42:2[2)]	Total SM
C6 (C4:1-DC)[2)]	PC ae C44:4	PC aa C42:4[2)]	Total SM/Total (SM+PC)
C6:1[1) 2)]	PC ae C44:5	PC aa C42:5	Total SM/Total PC
C7-DC[2)]	PC ae C44:6[2)]	PC aa C42:6	Total SM-non-OH
C8			Total SM-OH
C9[2)]			Total SM-OH/Total SM-non-OH
			Tyr/Phe
			SDMA/Arg
			Total DMA/Arg
Amino Acids	**Biogenic Amines**	**Sphingolipids**	**LysoPCs**
Ala	Ac-Orn	SM (OH) C14:1	lysoPC a C14:0[1) 3)]
Arg	ADMA	SM (OH) C16:1	lysoPC a C16:0
Asn	alpha-AAA	SM (OH) C22:1	lysoPC a C16:1
Asp	t4-OH-Pro[1) 2) 3)]	SM (OH) C22:2	lysoPC a C17:0
Cit	c4-OH-Pro[1) 2) 3)]	SM (OH) C24:1	lysoPC a C18:0
Gln	Carnosine[1) 2) 3)]	SM C16:0	lysoPC a C18:1
Glu	Creatinine	SM C16:1	lysoPC a C18:2
Gly	DOPA[1) 2) 3)]	SM C18:0	lysoPC a C20:3
His	Dopamine[1) 2) 3)]	SM C18:1	lysoPC a C20:4
Ile	Histamine[1) 2) 3)]	SM C20:2	lysoPC a C24:0[1) 3)]
Leu	Kynurenine	SM C22:3[1) 2) 3)]	lysoPC a C26:0[1) 2)]
Lys	Met-SO	SM C24:0	lysoPC a C26:1[1) 2) 3)]
Met	Nitro-Tyr[1) 2) 3)]	SM C24:1	lysoPC a C28:0
Orn	PEA[1) 2) 3)]	SM C26:0	lysoPC a C28:1[2)]
Phe	Putrescine [2)]	SM C26:1	
Pro	SDMA[2)]		
Ser	Serotonin[2)]		
Thr	Spermidine		
Trp	Spermine[1) 2)]		
Tyr	Taurine		
Val	total DMA		
	Hexose		
	H1		

SUPPLEMENTARY TABLE 2. Effect size and mean concentrations for each metabolite in plasma samples.

Given are minimum absolute differences in concentration [µM] for each metabolite in selected settings that are detectable given the sample size of 20, the observed variance in metabolite concentration changes, a power of 0.8 and a significance level of 0.01. Mean metabolite concentrations [µM] for plasma baseline are given as a comparison.

	Minimum detectable effect size		Baseline mean concentration
Metabolite	**0 vs. 24h on CP**	**0 vs. 24h at RT**	
C0	6.5031	5.8746	32.64
C10	0.0765	0.0855	0.33
C10:1	0.0558	0.0568	0.13
C10:2	0.0218	0.0231	0.04
C12	0.0333	0.0411	0.11
C14*	0.0168	0.0287	0.04
C14:1	0.0186	0.0348	0.10
C14:1-OH*	0.0100	0.0075	0.01
C14:2	0.0093	0.0152	0.03
C14:2-OH*	0.0095	0.0088	0.01
C16	0.0319	0.0827	0.08
C16:2*	0.0056	0.0077	0.01
C18*	0.0244	0.0346	0.03
C18:1	0.0653	0.1784	0.15
C18:2	0.0381	0.1361	0.06
C2	1.0642	1.2721	6.11
C3	0.0909	0.0959	0.34
C3-OH	0.0265	0.0316	0.06
C4	0.0580	0.0725	0.20
C4:1	0.0139	0.0089	0.02
C5	0.0529	0.0372	0.13
C5:1	0.0178	0.0193	0.04
C5:1-DC*	0.0091	0.0073	0.01
C6 (C4:1-DC)	0.0250	0.0332	0.08
C7-DC*	0.0136	0.0159	0.03
C8	0.0476	0.0364	0.18
C9*	0.0232	0.0174	0.05
Ala	72.7369	66.6126	354.15
Arg	22.6193	29.9383	79.83
Asn	12.0343	18.2343	50.75
Asp	2.9372	4.4873	6.19
Cit	6.5792	5.4782	29.48
Gln	154.3814	187.8147	584.91

Glu	14.5561	28.0155	35.49
Gly	48.7657	58.6801	254.29
His	21.3929	21.1822	85.94
Ile	15.8288	15.3568	72.50
Leu	30.8909	30.1909	146.30
Lys	25.6668	42.6487	138.46
Met	7.8345	6.1098	24.44
Orn	22.9340	39.0151	49.78
Phe	14.1803	13.2322	63.91
Pro	45.1560	41.0029	188.48
Ser	35.6120	36.2726	106.52
Thr	24.2056	25.5816	132.63
Trp	18.3746	12.4738	65.40
Tyr	15.8580	16.4620	65.97
Val	47.9659	49.2119	227.92
Acetylornithine	0.4283	0.4199	1.91
ADMA	0.2012	0.1708	0.46
alpha-AAA	0.4174	0.4749	0.68
Creatinine	9.1240	5.5114	71.93
Kynurenine	1.1670	1.1905	2.72
Met-SO	0.3820	0.3040	0.60
Putrescine*	0.0665	0.0585	0.12
SDMA*	0.3044	0.3138	0.38
Serotonin*	0.5236	0.3343	0.31
Spermidine*	0.1116	0.3429	0.25
Taurine	29.7456	49.4420	61.36
total dimethylarginine (DMA)	0.3451	0.3698	0.84
lysoPC a C16:0	19.9257	40.7909	90.11
lysoPC a C16:1	0.6500	1.5917	2.87
lysoPC a C17:0	0.4887	1.0079	1.92
lysoPC a C18:0	4.7097	12.2392	26.00
lysoPC a C18:1	4.3111	6.6186	22.47
lysoPC a C18:2	7.0036	9.0801	37.60
lysoPC a C20:3	0.6367	0.9574	2.76
lysoPC a C20:4	1.4631	2.3684	6.98
lysoPC a C28:0	0.1660	0.1538	0.36
lysoPC a C28:1	0.1841	0.2217	0.51
PC aa C24:0*	0.0420	0.0469	0.10
PC aa C28:1	0.5829	0.5245	2.92
PC aa C30:0	1.2002	1.4030	4.84
PC aa C32:0	2.9365	2.9729	14.72
PC aa C32:1	4.8465	8.8571	18.25
PC aa C32:2	1.1471	1.2491	5.20
PC aa C32:3	0.1758	0.1389	0.61
PC aa C34:1	34.4427	41.8075	221.73

PC aa C34:2	83.5219	102.6392	495.88
PC aa C34:3	4.0502	4.1085	21.14
PC aa C34:4	0.5312	0.5663	2.53
PC aa C36:0	0.5772	0.5232	3.13
PC aa C36:1	7.8376	8.2059	43.30
PC aa C36:2	48.9236	55.4820	277.70
PC aa C36:3	29.3523	33.8655	164.60
PC aa C36:4	39.5213	43.1230	225.50
PC aa C36:5	8.1456	9.8663	33.22
PC aa C36:6	0.3259	0.3292	1.35
PC aa C38:0	0.5636	0.6245	2.97
PC aa C38:1*	0.6426	0.5575	1.25
PC aa C38:3	7.8077	8.5925	45.74
PC aa C38:4	17.0339	18.6697	96.83
PC aa C38:5	9.7890	11.9756	57.01
PC aa C38:6	15.7775	16.2769	84.37
PC aa C40:1	0.1409	0.1229	0.41
PC aa C40:2	0.1686	0.1787	0.36
PC aa C40:3	0.2646	0.1821	0.66
PC aa C40:4	0.5924	0.7061	3.48
PC aa C40:5	1.7709	2.1319	10.50
PC aa C40:6	4.5686	4.8612	25.31
PC aa C42:0	0.1129	0.1171	0.48
PC aa C42:1	0.0781	0.1067	0.22
PC aa C42:2	0.0545	0.0629	0.20
PC aa C42:4	0.0723	0.0696	0.15
PC aa C42:5	0.1410	0.1528	0.47
PC aa C42:6	0.1663	0.1985	0.70
PC ae C30:0	0.1325	0.1096	0.43
PC ae C30:1*	0.2165	0.1315	0.07
PC ae C32:1	0.4602	0.6077	3.15
PC ae C32:2	0.1664	0.2341	0.80
PC ae C34:0	0.4511	0.4353	1.78
PC ae C34:1	1.9200	2.1297	11.40
PC ae C34:2	2.3994	2.1643	12.91
PC ae C34:3	1.2995	1.5672	8.14
PC ae C36:0	0.2586	0.2236	0.88
PC ae C36:1	1.6468	1.9245	9.29
PC ae C36:2	2.6011	3.3808	16.33
PC ae C36:3	1.1135	1.6650	7.75
PC ae C36:4	3.2587	3.5695	17.52
PC ae C36:5	1.8236	1.9923	11.13
PC ae C38:0	0.5245	0.6209	2.15
PC ae C38:1	0.4163	0.4517	0.26
PC ae C38:2	0.6743	0.8014	2.22

PC ae C38:3	0.7660	0.9134	4.32
PC ae C38:4	2.7376	3.0509	14.14
PC ae C38:5	2.9366	2.9181	16.87
PC ae C38:6	1.4081	1.5681	7.46
PC ae C40:1	0.4408	0.5037	1.55
PC ae C40:2	0.3508	0.4384	1.99
PC ae C40:3	0.3468	0.3202	1.13
PC ae C40:4	0.4775	0.6482	2.58
PC ae C40:5	0.7259	0.8220	3.69
PC ae C40:6	0.9799	1.0740	5.29
PC ae C42:0	0.2197	0.2117	0.65
PC ae C42:1	0.1236	0.0993	0.43
PC ae C42:2	0.2484	0.2170	0.66
PC ae C42:3	0.2708	0.2508	0.92
PC ae C42:4	0.2223	0.2693	0.93
PC ae C42:5	0.5164	0.4090	2.17
PC ae C44:3	0.0411	0.0660	0.12
PC ae C44:4	0.1342	0.1234	0.38
PC ae C44:5	0.3501	0.3384	1.65
PC ae C44:6	0.2432	0.2762	1.05
SM (OH) C14:1	1.6368	1.3833	8.01
SM (OH) C16:1	0.8390	0.7385	3.62
SM (OH) C22:1	3.4439	3.0080	12.89
SM (OH) C22:2	2.9432	2.3100	11.35
SM (OH) C24:1	0.3509	0.3768	1.13
SM C16:0	21.5329	21.6543	108.05
SM C16:1	3.4529	3.1695	16.48
SM C18:0	4.2296	4.2364	21.10
SM C18:1	2.2492	2.0944	10.49
SM C20:2	0.1668	0.2313	0.39
SM C24:0	4.1482	4.1605	17.59
SM C24:1	10.6057	10.0133	46.36
SM C26:0*	0.1297	0.1253	0.21
SM C26:1	0.1546	0.1588	0.36
Hexose	729.3056	860.3496	5128.19

SUPPLEMENTARY TABLE 3. ICC, WCV and BCV with confidence intervals in plasma and serum metabolites.

	Plasma						Serum					
Metabolite	**WCV**	**CI**	**BCV**	**CI**	**ICC**	**CI**	**WCV**	**CI**	**BCV**	**CI**	**ICC**	**CI**
C0	0.10	0.08-0.12	0.25	0.22-0.30	0.87	0.81-0.92	0.11	0.09-0.13	0.27	0.22-0.35	0.86	0.76-0.93
C10	0.25	0.22-0.31	0.37	0.27-0.49	0.67	0.51-0.81	0.28	0.22-0.36	0.33	0.24-0.46	0.59	0.34-0.80
C10:1	0.28	0.23-0.34	0.27	0.17-0.38	0.48	0.20-0.69	0.28	0.22-0.35	0.29	0.18-0.44	0.52	0.22-0.80
C10:2	0.27	0.23-0.32	0.24	0.09-0.40	0.44	0.06-0.74	0.26	0.19-0.35	0.29	0.20-0.40	0.56	0.47-0.70
C12	0.30	0.24-0.41	0.28	0.18-0.38	0.45	0.18-0.69	0.30	0.24-0.37	0.27	0.17-0.42	0.45	0.19-0.75
C14*	0.24	0.19-0.30	0.16	0.07-0.26	0.32	0.04-0.61	0.23	0.17-0.30	0.14	0.06-0.20	0.28	0.00-0.52
C14:1	0.38	0.31-0.45	0.27	0.19-0.37	0.33	0.19-0.47	0.30	0.24-0.37	0.25	0.17-0.35	0.41	0.19-0.63
C14:1-OH*	0.35	0.30-0.43	0.00	0.00-0.14	0.00	0.00-0.16	0.37	0.32-0.43	0.16	0.00-0.29	0.15	0.00-0.44
C14:2	0.40	0.33-0.47	0.32	0.22-0.46	0.39	0.18-0.60	0.35	0.29-0.42	0.33	0.21-0.48	0.46	0.20-0.70
C14:2-OH*	0.38	0.31-0.46	0.24	0.00-0.44	0.29	0.00-0.64	0.33	0.26-0.41	0.14	0.00-0.27	0.16	0.00-0.48
C16	0.23	0.19-0.29	0.09	0.00-0.18	0.13	0.00-0.45	0.19	0.15-0.24	0.12	0.03-0.19	0.29	0.00-0.61
C16:2*	0.45	0.36-0.53	0.14	0.00-0.27	0.09	0.00-0.33	0.40	0.32-0.48	0.20	0.04-0.31	0.20	0.00-0.45
C18*	0.28	0.21-0.35	0.21	0.10-0.32	0.36	0.05-0.68	0.33	0.25-0.48	0.25	0.10-0.35	0.36	0.00-0.62
C18:1	0.20	0.16-0.25	0.17	0.10-0.25	0.43	0.17-0.68	0.16	0.13-0.20	0.17	0.11-0.24	0.54	0.26-0.77
C18:2	0.26	0.21-0.34	0.21	0.11-0.29	0.39	0.15-0.55	0.24	0.20-0.29	0.24	0.16-0.33	0.50	0.26-0.66
C2	0.22	0.18-0.28	0.27	0.18-0.41	0.61	0.34-0.82	0.19	0.14-0.23	0.29	0.19-0.44	0.71	0.47-0.90
C3	0.15	0.11-0.20	0.26	0.19-0.35	0.76	0.66-0.87	0.14	0.12-0.17	0.24	0.18-0.33	0.73	0.59-0.85
C3-OH	0.21	0.15-0.29	0.00	0.00-0.08	0.00	0.00-0.20	0.15	0.12-0.17	0.06	0.00-0.13	0.16	0.00-0.51
C4	0.15	0.12-0.17	0.29	0.22-0.38	0.80	0.68-0.89	0.18	0.15-0.22	0.30	0.22-0.42	0.74	0.55-0.87
C4:1	0.20	0.16-0.24	0.17	0.11-0.26	0.41	0.21-0.64	0.19	0.15-0.26	0.13	0.05-0.19	0.31	0.00-0.56
C5	0.18	0.15-0.22	0.26	0.19-0.34	0.67	0.49-0.80	0.20	0.17-0.23	0.30	0.22-0.39	0.70	0.52-0.81
C5:1	0.21	0.16-0.30	0.11	0.05-0.16	0.21	0.01-0.43	0.20	0.16-0.24	0.13	0.06-0.19	0.30	0.04-0.54
C5:1-DC*	0.31	0.25-0.41	0.34	0.21-0.51	0.55	0.24-0.78	0.34	0.27-0.42	0.14	0.00-0.34	0.15	0.00-0.60
C6 (C4:1-DC)	0.24	0.18-0.30	0.22	0.16-0.30	0.45	0.25-0.71	0.25	0.20-0.33	0.27	0.19-0.37	0.55	0.26-0.76
C7-DC*	0.36	0.30-0.47	0.29	0.18-0.43	0.39	0.15-0.59	0.30	0.25-0.38	0.28	0.20-0.40	0.47	0.27-0.67
C8	0.24	0.19-0.29	0.31	0.22-0.43	0.63	0.42-0.81	0.25	0.21-0.30	0.29	0.21-0.38	0.57	0.36-0.73
C9*	0.30	0.24-0.40	0.37	0.26-0.54	0.60	0.49-0.70	0.43	0.27-0.62	0.44	0.30-0.64	0.52	0.38-0.72
Ala	0.12	0.10-0.14	0.21	0.17-0.28	0.76	0.63-0.85	0.13	0.10-0.17	0.22	0.17-0.29	0.74	0.58-0.87
Arg	0.16	0.13-0.21	0.20	0.15-0.27	0.60	0.36-0.76	0.14	0.10-0.17	0.20	0.15-0.26	0.67	0.45-0.85
Asn	0.12	0.10-0.15	0.16	0.12-0.22	0.63	0.41-0.77	0.13	0.10-0.17	0.19	0.13-0.27	0.67	0.39-0.88
Asp	0.24	0.19-0.32	0.33	0.25-0.44	0.65	0.40-0.80	0.21	0.16-0.28	0.20	0.15-0.27	0.49	0.27-0.70
Cit	0.13	0.09-0.18	0.20	0.14-0.28	0.72	0.43-0.90	0.13	0.10-0.17	0.20	0.15-0.27	0.69	0.55-0.82
Gln	0.10	0.08-0.14	0.13	0.11-0.17	0.65	0.50-0.82	0.12	0.10-0.15	0.13	0.09-0.18	0.53	0.30-0.76
Glu	0.34	0.27-0.45	0.41	0.28-0.58	0.59	0.30-0.78	0.28	0.22-0.36	0.36	0.25-0.48	0.62	0.33-0.82
Gly	0.13	0.10-0.16	0.20	0.14-0.30	0.72	0.48-0.87	0.12	0.10-0.14	0.22	0.16-0.32	0.77	0.59-0.89
His	0.08	0.06-0.09	0.09	0.06-0.14	0.60	0.36-0.78	0.09	0.07-0.13	0.11	0.07-0.16	0.58	0.30-0.79
Ile	0.11	0.09-0.12	0.16	0.12-0.22	0.69	0.57-0.80	0.12	0.09-0.15	0.19	0.14-0.25	0.70	0.50-0.87
Leu	0.08	0.06-0.10	0.15	0.12-0.18	0.77	0.67-0.86	0.11	0.09-0.13	0.15	0.12-0.20	0.67	0.49-0.81
Lys	0.11	0.09-0.14	0.11	0.07-0.17	0.50	0.23-0.71	0.12	0.09-0.15	0.11	0.07-0.18	0.49	0.20-0.78

Met	0.13	0.11-0.16	0.12	0.08-0.18	0.45	0.24-0.69	0.13	0.10-0.15	0.16	0.11-0.22	0.62	0.39-0.79
Orn	0.14	0.11-0.17	0.24	0.16-0.34	0.75	0.56-0.88	0.11	0.09-0.13	0.23	0.17-0.32	0.82	0.72-0.90
Phe	0.08	0.06-0.09	0.10	0.07-0.14	0.60	0.37-0.81	0.08	0.07-0.10	0.12	0.08-0.18	0.67	0.44-0.86
Pro	0.16	0.12-0.20	0.31	0.21-0.46	0.79	0.60-0.95	0.15	0.11-0.19	0.33	0.21-0.49	0.84	0.63-0.97
Ser	0.12	0.09-0.17	0.13	0.08-0.19	0.54	0.21-0.80	0.12	0.10-0.16	0.10	0.04-0.18	0.41	0.01-0.74
Thr	0.17	0.14-0.24	0.21	0.15-0.28	0.60	0.38-0.76	0.21	0.14-0.32	0.20	0.14-0.27	0.47	0.22-0.74
Trp	0.13	0.10-0.15	0.15	0.09-0.23	0.60	0.29-0.83	0.12	0.09-0.15	0.18	0.12-0.27	0.68	0.45-0.91
Tyr	0.11	0.09-0.14	0.18	0.15-0.23	0.74	0.59-0.83	0.14	0.12-0.17	0.20	0.15-0.27	0.66	0.46-0.82
Val	0.11	0.09-0.14	0.13	0.10-0.17	0.59	0.39-0.76	0.12	0.10-0.15	0.15	0.12-0.20	0.63	0.42-0.77
Acetylornithine	1.22	0.27-1.61	1.62	0.67-2.20	0.64	0.56-0.86	1.09	0.21-1.40	1.56	0.64-2.04	0.67	0.59-0.89
ADMA	0.12	0.09-0.15	0.12	0.04-0.20	0.48	0.10-0.83	0.20	0.16-0.24	0.05	0.00-0.13	0.05	0.00-0.38
alpha-AAA	0.31	0.21-0.45	0.35	0.26-0.48	0.57	0.36-0.80	0.31	0.21-0.44	0.43	0.30-0.62	0.66	0.45-0.87
Creatinine	0.05	0.04-0.07	0.16	0.13-0.21	0.90	0.84-0.95	0.11	0.08-0.19	0.17	0.13-0.22	0.68	0.35-0.86
Kynurenine	0.22	0.16-0.32	0.19	0.12-0.29	0.43	0.11-0.70	0.15	0.12-0.18	0.23	0.18-0.31	0.70	0.55-0.86
Met-SO	0.29	0.22-0.39	0.16	0.00-0.35	0.24	0.00-0.62	0.19	0.15-0.24	0.27	0.19-0.41	0.68	0.46-0.83
Putrescine*	0.29	0.22-0.39	0.31	0.22-0.42	0.54	0.24-0.75	0.23	0.18-0.30	0.31	0.22-0.42	0.63	0.44-0.79
SDMA*	0.29	0.21-0.40	0.21	0.09-0.35	0.35	0.01-0.68	0.26	0.21-0.35	0.22	0.13-0.34	0.41	0.15-0.60
Serotonin*	0.36	0.25-0.53	0.44	0.29-0.67	0.61	0.25-0.84	0.24	0.19-0.29	0.54	0.42-0.72	0.84	0.76-0.91
Spermidine*	0.15	0.12-0.20	0.26	0.21-0.33	0.76	0.59-0.85	0.12	0.10-0.18	0.32	0.26-0.40	0.87	0.77-0.93
Taurine	0.13	0.10-0.17	0.22	0.17-0.30	0.76	0.58-0.88	0.16	0.13-0.21	0.18	0.14-0.23	0.56	0.37-0.73
total DMA	0.16	0.11-0.24	0.15	0.08-0.22	0.48	0.03-0.78	0.14	0.11-0.18	0.17	0.14-0.21	0.61	0.43-0.78
lysoPC a C16:0	0.14	0.11-0.17	0.16	0.11-0.22	0.58	0.33-0.72	0.14	0.11-0.17	0.17	0.10-0.24	0.59	0.27-0.81
lysoPC a C16:1	0.19	0.16-0.25	0.16	0.09-0.24	0.42	0.19-0.67	0.20	0.14-0.27	0.20	0.07-0.29	0.51	0.14-0.83
lysoPC a C17:0	0.14	0.11-0.16	0.24	0.20-0.29	0.76	0.66-0.84	0.15	0.12-0.19	0.22	0.16-0.28	0.67	0.44-0.84
lysoPC a C18:0	0.15	0.13-0.19	0.24	0.18-0.34	0.71	0.57-0.84	0.15	0.12-0.18	0.24	0.17-0.35	0.74	0.53-0.89
lysoPC a C18:1	0.19	0.15-0.24	0.29	0.22-0.39	0.71	0.58-0.84	0.17	0.14-0.21	0.31	0.21-0.41	0.77	0.54-0.89
lysoPC a C18:2	0.26	0.19-0.37	0.37	0.28-0.49	0.67	0.48-0.78	0.21	0.18-0.28	0.38	0.29-0.53	0.76	0.62-0.87
lysoPC a C20:3	0.18	0.15-0.22	0.20	0.13-0.30	0.56	0.32-0.75	0.17	0.14-0.21	0.25	0.17-0.35	0.67	0.45-0.83
lysoPC a C20:4	0.15	0.11-0.21	0.34	0.26-0.43	0.83	0.66-0.93	0.15	0.12-0.17	0.33	0.25-0.45	0.83	0.72-0.90
lysoPC a C28:0	0.20	0.16-0.26	0.05	0.00-0.13	0.07	0.00-0.36	0.17	0.14-0.21	0.10	0.05-0.16	0.28	0.05-0.52
lysoPC a C28:1	0.16	0.14-0.19	0.18	0.14-0.23	0.55	0.42-0.68	0.14	0.11-0.17	0.20	0.13-0.32	0.69	0.43-0.88
PC aa C24:0*	0.17	0.14-0.20	0.14	0.07-0.21	0.42	0.14-0.64	0.17	0.14-0.23	0.13	0.08-0.20	0.37	0.17-0.55
PC aa C28:1	0.09	0.08-0.11	0.18	0.13-0.26	0.81	0.69-0.91	0.10	0.07-0.12	0.19	0.13-0.30	0.80	0.61-0.92
PC aa C30:0	0.23	0.18-0.30	0.26	0.16-0.40	0.56	0.30-0.86	0.23	0.18-0.29	0.30	0.18-0.45	0.63	0.35-0.89
PC aa C32:0	0.13	0.10-0.15	0.21	0.14-0.29	0.73	0.51-0.90	0.11	0.09-0.13	0.21	0.15-0.29	0.79	0.65-0.92
PC aa C32:1	0.31	0.24-0.38	0.55	0.26-0.81	0.76	0.46-0.85	0.28	0.23-0.34	0.57	0.30-0.82	0.80	0.63-0.86
PC aa C32:2	0.19	0.15-0.24	0.39	0.32-0.51	0.80	0.70-0.92	0.20	0.16-0.25	0.40	0.32-0.51	0.79	0.70-0.92
PC aa C32:3	0.11	0.08-0.15	0.22	0.15-0.32	0.80	0.55-0.93	0.19	0.14-0.25	0.23	0.15-0.34	0.59	0.37-0.83
PC aa C34:1	0.16	0.12-0.20	0.23	0.11-0.37	0.69	0.32-0.89	0.15	0.12-0.19	0.26	0.15-0.41	0.74	0.51-0.86
PC aa C34:2	0.12	0.10-0.15	0.17	0.12-0.23	0.67	0.43-0.81	0.10	0.08-0.13	0.19	0.14-0.26	0.80	0.64-0.91
PC aa C34:3	0.14	0.12-0.17	0.30	0.21-0.40	0.81	0.65-0.90	0.15	0.11-0.19	0.31	0.23-0.42	0.81	0.65-0.93
PC aa C34:4	0.17	0.13-0.24	0.39	0.28-0.54	0.84	0.65-0.95	0.21	0.15-0.31	0.39	0.29-0.54	0.77	0.54-0.92
PC aa C36:0	0.12	0.09-0.16	0.22	0.18-0.28	0.78	0.63-0.90	0.12	0.10-0.15	0.24	0.19-0.29	0.79	0.64-0.88
PC aa C36:1	0.16	0.12-0.20	0.17	0.10-0.27	0.52	0.27-0.70	0.16	0.12-0.20	0.20	0.13-0.31	0.62	0.39-0.78
PC aa C36:2	0.11	0.09-0.15	0.14	0.11-0.19	0.62	0.38-0.78	0.10	0.07-0.13	0.16	0.12-0.22	0.73	0.53-0.88

PC aa C36:3	0.11	0.09-0.13	0.21	0.16-0.30	0.80	0.65-0.91	0.12	0.09-0.16	0.24	0.18-0.33	0.81	0.65-0.93
PC aa C36:4	0.09	0.07-0.12	0.22	0.16-0.31	0.85	0.73-0.94	0.11	0.08-0.15	0.23	0.17-0.32	0.81	0.63-0.94
PC aa C36:5	0.39	0.23-0.65	0.36	0.23-0.57	0.46	0.06-0.74	0.47	0.20-0.70	0.38	0.23-0.61	0.40	0.04-0.69
PC aa C36:6	0.24	0.15-0.40	0.43	0.33-0.61	0.76	0.46-0.92	0.28	0.15-0.41	0.42	0.32-0.61	0.70	0.43-0.93
PC aa C38:0	0.13	0.10-0.16	0.27	0.21-0.37	0.81	0.66-0.89	0.15	0.12-0.20	0.28	0.22-0.37	0.78	0.67-0.90
PC aa C38:1*	0.26	0.23-0.30	0.31	0.18-0.46	0.58	0.32-0.75	0.23	0.20-0.27	0.27	0.19-0.41	0.59	0.38-0.79
PC aa C38:3	0.11	0.08-0.15	0.21	0.14-0.30	0.77	0.58-0.87	0.12	0.10-0.16	0.23	0.16-0.34	0.77	0.62-0.85
PC aa C38:4	0.08	0.06-0.11	0.17	0.12-0.24	0.80	0.61-0.91	0.12	0.10-0.14	0.16	0.11-0.24	0.65	0.40-0.83
PC aa C38:5	0.12	0.10-0.15	0.19	0.14-0.25	0.71	0.53-0.84	0.15	0.11-0.19	0.20	0.15-0.27	0.65	0.50-0.82
PC aa C38:6	0.13	0.11-0.18	0.36	0.27-0.50	0.88	0.75-0.95	0.15	0.12-0.18	0.35	0.26-0.49	0.85	0.72-0.94
PC aa C40:1	0.17	0.12-0.23	0.21	0.16-0.30	0.63	0.35-0.87	0.17	0.13-0.25	0.20	0.15-0.24	0.57	0.37-0.78
PC aa C40:2	0.30	0.19-0.43	0.19	0.13-0.27	0.29	0.17-0.59	0.23	0.18-0.31	0.25	0.19-0.34	0.55	0.41-0.73
PC aa C40:3	0.20	0.17-0.25	0.20	0.15-0.27	0.48	0.36-0.61	0.22	0.13-0.35	0.24	0.18-0.33	0.55	0.29-0.74
PC aa C40:4	0.11	0.09-0.15	0.22	0.12-0.34	0.78	0.61-0.87	0.13	0.11-0.17	0.24	0.14-0.37	0.76	0.56-0.86
PC aa C40:5	0.11	0.09-0.13	0.24	0.17-0.32	0.83	0.70-0.92	0.12	0.09-0.15	0.24	0.18-0.33	0.80	0.63-0.89
PC aa C40:6	0.13	0.10-0.17	0.35	0.27-0.46	0.88	0.79-0.93	0.14	0.11-0.18	0.35	0.26-0.47	0.85	0.76-0.92
PC aa C42:0	0.15	0.12-0.21	0.23	0.17-0.33	0.70	0.46-0.87	0.12	0.10-0.15	0.23	0.17-0.32	0.79	0.63-0.89
PC aa C42:1	0.20	0.17-0.23	0.21	0.15-0.30	0.51	0.32-0.71	0.18	0.15-0.22	0.20	0.14-0.28	0.56	0.33-0.76
PC aa C42:2	0.21	0.16-0.32	0.20	0.13-0.27	0.46	0.08-0.69	0.21	0.15-0.29	0.23	0.16-0.33	0.55	0.31-0.85
PC aa C42:4	0.19	0.15-0.22	0.24	0.17-0.33	0.61	0.40-0.77	0.23	0.19-0.31	0.16	0.05-0.24	0.31	0.00-0.59
PC aa C42:5	0.24	0.18-0.30	0.34	0.24-0.48	0.66	0.48-0.79	0.29	0.17-0.43	0.33	0.25-0.47	0.57	0.31-0.74
PC aa C42:6	0.16	0.12-0.23	0.21	0.16-0.29	0.63	0.35-0.80	0.21	0.12-0.31	0.23	0.17-0.30	0.54	0.25-0.78
PC ae C30:0	0.16	0.13-0.19	0.17	0.11-0.25	0.53	0.28-0.75	0.15	0.12-0.19	0.21	0.15-0.28	0.66	0.48-0.83
PC ae C30:1*	1.18	0.93-1.51	0.00	0.00-0.47	0.00	0.00-0.17	0.87	0.67-1.15	0.53	0.00-0.95	0.27	0.00-0.61
PC ae C32:1	0.11	0.09-0.16	0.16	0.12-0.23	0.67	0.42-0.85	0.12	0.09-0.16	0.19	0.13-0.26	0.71	0.44-0.83
PC ae C32:2	0.12	0.09-0.15	0.19	0.15-0.25	0.73	0.53-0.87	0.12	0.10-0.16	0.20	0.14-0.26	0.72	0.52-0.86
PC ae C34:0	0.16	0.14-0.19	0.19	0.13-0.28	0.57	0.34-0.78	0.17	0.14-0.19	0.21	0.14-0.32	0.61	0.42-0.77
PC ae C34:1	0.13	0.11-0.16	0.17	0.11-0.27	0.64	0.38-0.83	0.14	0.10-0.17	0.20	0.14-0.29	0.67	0.47-0.81
PC ae C34:2	0.15	0.11-0.21	0.19	0.11-0.28	0.61	0.41-0.77	0.13	0.10-0.17	0.20	0.12-0.30	0.71	0.48-0.83
PC ae C34:3	0.13	0.10-0.15	0.19	0.14-0.25	0.68	0.53-0.83	0.09	0.07-0.12	0.19	0.15-0.24	0.83	0.65-0.91
PC ae C36:0	0.12	0.10-0.18	0.18	0.13-0.24	0.68	0.37-0.83	0.12	0.10-0.14	0.18	0.13-0.25	0.70	0.48-0.85
PC ae C36:1	0.13	0.11-0.16	0.15	0.09-0.22	0.56	0.30-0.75	0.13	0.10-0.16	0.16	0.10-0.24	0.62	0.37-0.79
PC ae C36:2	0.11	0.09-0.14	0.15	0.11-0.20	0.64	0.43-0.79	0.10	0.08-0.14	0.16	0.12-0.22	0.72	0.52-0.86
PC ae C36:3	0.12	0.10-0.15	0.16	0.10-0.22	0.63	0.40-0.78	0.12	0.09-0.15	0.19	0.12-0.26	0.72	0.40-0.87
PC ae C36:4	0.12	0.09-0.14	0.18	0.13-0.26	0.72	0.56-0.87	0.11	0.09-0.14	0.20	0.15-0.27	0.75	0.55-0.89
PC ae C36:5	0.12	0.09-0.15	0.22	0.16-0.32	0.78	0.59-0.91	0.12	0.09-0.17	0.26	0.18-0.36	0.81	0.71-0.92
PC ae C38:0	0.22	0.15-0.34	0.29	0.22-0.37	0.64	0.35-0.82	0.25	0.18-0.37	0.29	0.22-0.38	0.57	0.31-0.76
PC ae C38:1	0.78	0.55-1.14	0.57	0.23-0.92	0.35	0.00-0.64	0.72	0.53-0.99	0.74	0.53-1.04	0.51	0.31-0.69
PC ae C38:2	0.16	0.13-0.19	0.19	0.12-0.25	0.58	0.30-0.80	0.13	0.10-0.17	0.19	0.13-0.27	0.69	0.41-0.88
PC ae C38:3	0.10	0.08-0.13	0.17	0.13-0.24	0.74	0.56-0.89	0.12	0.09-0.16	0.19	0.14-0.28	0.73	0.48-0.87
PC ae C38:4	0.09	0.07-0.11	0.09	0.06-0.15	0.55	0.25-0.80	0.10	0.08-0.13	0.10	0.06-0.14	0.48	0.19-0.74
PC ae C38:5	0.09	0.08-0.11	0.15	0.11-0.21	0.73	0.57-0.86	0.12	0.09-0.17	0.17	0.12-0.23	0.66	0.46-0.84
PC ae C38:6	0.14	0.10-0.18	0.26	0.20-0.36	0.79	0.62-0.92	0.16	0.11-0.21	0.28	0.23-0.38	0.77	0.66-0.90
PC ae C40:1	0.18	0.14-0.22	0.19	0.13-0.26	0.53	0.31-0.75	0.18	0.14-0.23	0.20	0.16-0.27	0.55	0.37-0.73
PC ae C40:2	0.10	0.09-0.11	0.20	0.16-0.26	0.81	0.74-0.88	0.10	0.09-0.14	0.19	0.15-0.27	0.78	0.63-0.88

PC ae C40:3	0.12	0.10-0.15	0.16	0.12-0.23	0.63	0.42-0.81	0.11	0.08-0.15	0.17	0.13-0.23	0.69	0.45-0.84
PC ae C40:4	0.10	0.08-0.13	0.13	0.10-0.18	0.64	0.42-0.83	0.11	0.09-0.14	0.13	0.09-0.19	0.61	0.38-0.76
PC ae C40:5	0.11	0.08-0.13	0.13	0.09-0.17	0.59	0.31-0.77	0.11	0.08-0.13	0.14	0.10-0.18	0.65	0.47-0.74
PC ae C40:6	0.12	0.09-0.16	0.23	0.18-0.30	0.80	0.60-0.90	0.14	0.11-0.16	0.23	0.18-0.30	0.74	0.59-0.86
PC ae C42:0	0.15	0.11-0.21	0.24	0.19-0.32	0.72	0.64-0.83	0.13	0.09-0.18	0.24	0.17-0.31	0.77	0.43-0.86
PC ae C42:1	0.15	0.12-0.18	0.18	0.12-0.24	0.59	0.34-0.75	0.17	0.13-0.24	0.19	0.12-0.29	0.56	0.20-0.84
PC ae C42:2	0.15	0.12-0.18	0.20	0.12-0.30	0.64	0.36-0.87	0.15	0.12-0.17	0.23	0.15-0.34	0.71	0.49-0.89
PC ae C42:3	0.14	0.12-0.17	0.20	0.16-0.27	0.67	0.54-0.81	0.15	0.11-0.19	0.18	0.14-0.23	0.60	0.41-0.74
PC ae C42:4	0.13	0.11-0.15	0.14	0.10-0.18	0.54	0.40-0.67	0.13	0.10-0.18	0.12	0.08-0.16	0.44	0.17-0.65
PC ae C42:5	0.10	0.08-0.12	0.14	0.09-0.20	0.66	0.39-0.85	0.09	0.07-0.11	0.15	0.11-0.23	0.76	0.57-0.90
PC ae C44:3	0.24	0.20-0.28	0.12	0.01-0.18	0.22	0.00-0.41	0.24	0.21-0.28	0.15	0.06-0.24	0.28	0.05-0.50
PC ae C44:4	0.16	0.13-0.18	0.14	0.09-0.20	0.45	0.23-0.64	0.19	0.16-0.23	0.13	0.05-0.20	0.30	0.04-0.54
PC ae C44:5	0.12	0.10-0.16	0.16	0.10-0.23	0.62	0.35-0.80	0.11	0.09-0.14	0.15	0.09-0.22	0.63	0.33-0.82
PC ae C44:6	0.12	0.10-0.14	0.22	0.16-0.28	0.76	0.63-0.85	0.13	0.10-0.18	0.21	0.15-0.29	0.73	0.47-0.87
SM (OH) C14:1	0.10	0.08-0.14	0.15	0.11-0.21	0.68	0.44-0.83	0.10	0.08-0.12	0.11	0.07-0.16	0.57	0.36-0.73
SM (OH) C16:1	0.11	0.08-0.13	0.13	0.09-0.18	0.59	0.34-0.79	0.10	0.09-0.13	0.12	0.07-0.17	0.55	0.31-0.72
SM (OH) C22:1	0.10	0.09-0.13	0.16	0.11-0.21	0.70	0.49-0.84	0.11	0.09-0.16	0.16	0.12-0.23	0.68	0.45-0.82
SM (OH) C22:2	0.11	0.09-0.14	0.13	0.08-0.18	0.57	0.31-0.77	0.10	0.08-0.13	0.15	0.11-0.20	0.67	0.43-0.81
SM (OH) C24:1	0.14	0.11-0.19	0.12	0.08-0.17	0.41	0.18-0.63	0.15	0.12-0.19	0.11	0.07-0.15	0.35	0.16-0.55
SM C16:0	0.10	0.08-0.11	0.07	0.04-0.11	0.38	0.12-0.65	0.09	0.08-0.12	0.09	0.05-0.14	0.47	0.15-0.69
SM C16:1	0.09	0.08-0.12	0.15	0.10-0.21	0.72	0.47-0.87	0.09	0.07-0.12	0.15	0.11-0.21	0.73	0.61-0.83
SM C18:0	0.12	0.10-0.15	0.11	0.06-0.17	0.48	0.15-0.74	0.11	0.09-0.15	0.13	0.09-0.20	0.59	0.32-0.75
SM C18:1	0.11	0.09-0.13	0.16	0.10-0.24	0.69	0.42-0.87	0.12	0.09-0.14	0.17	0.12-0.24	0.68	0.50-0.77
SM C20:2	0.27	0.21-0.36	0.15	0.08-0.26	0.25	0.07-0.54	0.20	0.16-0.26	0.21	0.15-0.29	0.53	0.29-0.73
SM C24:0	0.10	0.09-0.12	0.18	0.10-0.28	0.74	0.46-0.88	0.12	0.09-0.16	0.21	0.12-0.33	0.77	0.47-0.91
SM C24:1	0.11	0.09-0.14	0.16	0.09-0.24	0.70	0.37-0.87	0.10	0.07-0.14	0.21	0.13-0.29	0.82	0.55-0.94
SM C26:0*	0.26	0.22-0.30	0.31	0.19-0.44	0.59	0.32-0.77	0.34	0.25-0.43	0.31	0.22-0.41	0.45	0.33-0.72
SM C26:1	0.19	0.15-0.22	0.16	0.10-0.23	0.41	0.22-0.63	0.19	0.15-0.24	0.21	0.13-0.34	0.55	0.27-0.78
Hexose	0.08	0.06-0.10	0.12	0.06-0.21	0.72	0.32-0.94	0.09	0.06-0.12	0.14	0.06-0.23	0.72	0.29-0.94

SUPPLEMENTARY TABLE 4. Comparison of the median metabolite concentrations in plasma and serum samples.

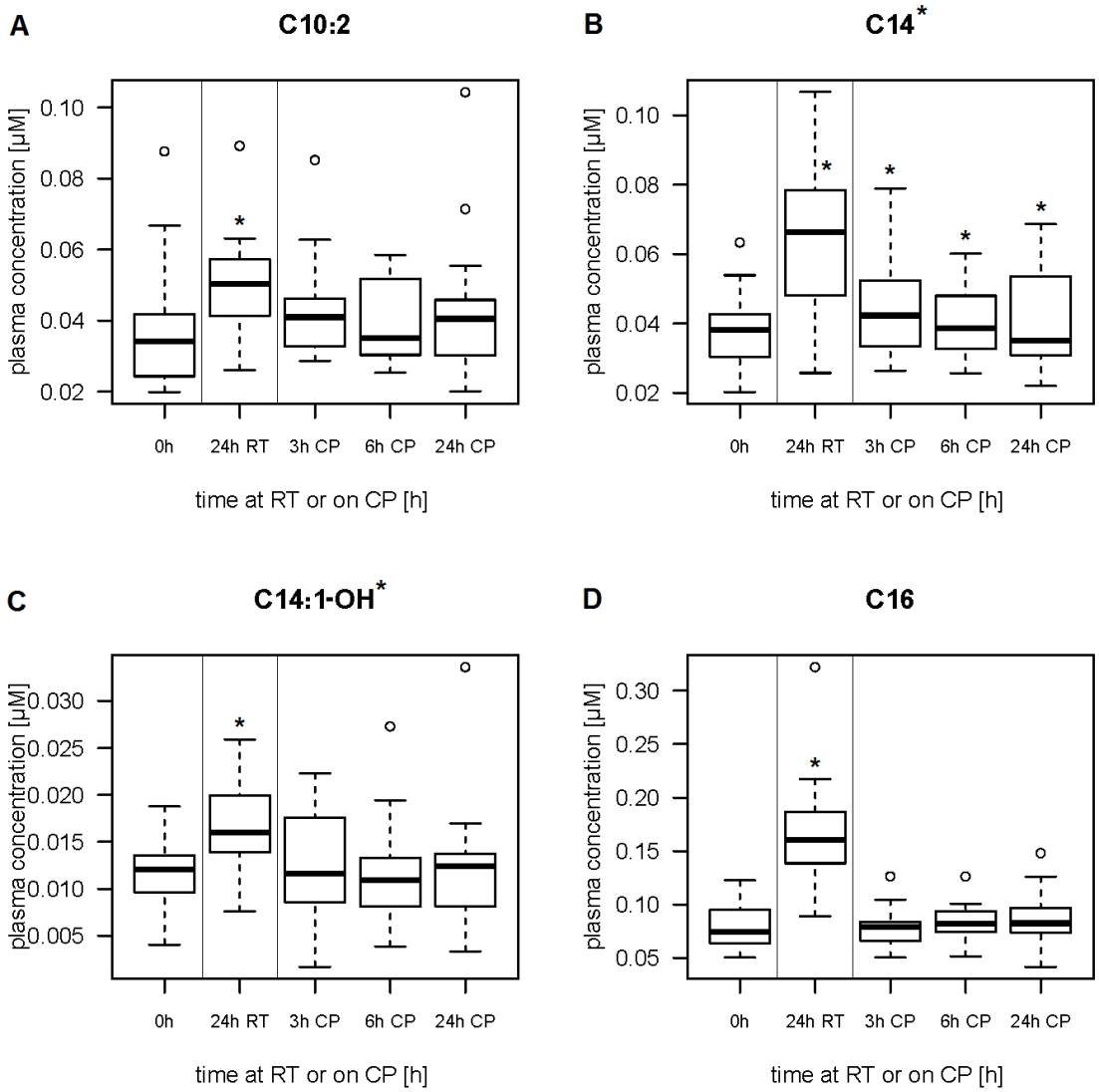
Serum W and plasma baseline samples that were collected on three days within 20 subjects were tested in a pairwise manner using Wilcoxon-signed rank test ($p < 0.01$).

Metabolite	p-value (Wilcoxon)	Metabolite	p-value (Wilcoxon)	Metabolite	p-value (Wilcoxon)
C0	3.61E-04	Acetylornithine	1.31E-01	PC ae C30:0	5.64E-04
C10	3.74E-01	ADMA	4.67E-01	PC ae C30:1*	4.14E-01
C10:1	2.40E-03	alpha-AAA	2.40E-01	PC ae C32:1	5.16E-04
C10:2	3.12E-03	Creatinine	2.03E-05	PC ae C32:2	1.38E-02
C12	1.42E-01	Kynurenine	2.78E-03	PC ae C34:0	7.13E-04
C14*	2.26E-02	Met-SO	6.97E-01	PC ae C34:1	7.55E-04
C14:1	1.99E-02	Putrescine*	1.99E-09	PC ae C34:2	2.74E-04
C14:1-OH*	7.69E-01	SDMA*	2.59E-01	PC ae C34:3	2.14E-04
C14:2	7.24E-03	Serotonin*	5.28E-11	PC ae C36:0	3.07E-02
C14:2-OH*	2.56E-01	Spermidine*	3.82E-01	PC ae C36:1	2.34E-03
C16	8.12E-05	Taurine	6.89E-11	PC ae C36:2	2.73E-05
C16:2*	8.91E-02	total DMA	3.93E-03	PC ae C36:3	4.28E-05
C18*	8.61E-01	lysoPC a C16:0	2.56E-06	PC ae C36:4	3.61E-04
C18:1	1.80E-06	lysoPC a C16:1	7.43E-06	PC ae C36:5	2.14E-04
C18:2	1.89E-03	lysoPC a C17:0	5.32E-04	PC ae C38:0	2.14E-04
C2	6.74E-03	lysoPC a C18:0	3.23E-06	PC ae C38:1	6.83E-01
C3	1.55E-01	lysoPC a C18:1	3.76E-06	PC ae C38:2	5.97E-02
C3-OH	1.19E-01	lysoPC a C18:2	6.64E-06	PC ae C38:3	1.66E-04
C4	2.22E-03	lysoPC a C20:3	7.21E-01	PC ae C38:4	7.11E-05
C4:1	7.81E-01	lysoPC a C20:4	9.27E-05	PC ae C38:5	1.06E-04
C5	1.12E-01	lysoPC a C28:0	2.80E-01	PC ae C38:6	1.98E-05
C5:1	6.97E-01	lysoPC a C28:1	3.13E-01	PC ae C40:1	3.74E-01
C5:1-DC*	9.94E-01	PC aa C24:0*	2.87E-01	PC ae C40:2	1.89E-03
C6 (C4:1-DC)	9.81E-01	PC aa C28:1	1.44E-05	PC ae C40:3	2.24E-01
C7-DC*	1.85E-01	PC aa C30:0	3.50E-04	PC ae C40:4	6.74E-03
C8	1.24E-05	PC aa C32:0	4.23E-03	PC ae C40:5	1.03E-03
C9*	1.87E-01	PC aa C32:1	1.00E-03	PC ae C40:6	6.22E-05
Ala	1.27E-07	PC aa C32:2	6.88E-05	PC ae C42:0	2.17E-02
Arg	2.41E-10	PC aa C32:3	4.72E-04	PC ae C42:1	4.79E-01
Asn	4.30E-06	PC aa C34:1	5.62E-05	PC ae C42:2	2.26E-02
Asp	5.57E-11	PC aa C34:2	7.35E-05	PC ae C42:3	3.74E-02
Cit	2.83E-02	PC aa C34:3	1.92E-05	PC ae C42:4	1.61E-04
Gln	8.26E-05	PC aa C34:4	3.46E-03	PC ae C42:5	3.10E-04
Glu	3.40E-01	PC aa C36:0	1.15E-02	PC ae C44:3	8.24E-01
Gly	1.22E-07	PC aa C36:1	5.16E-04	PC ae C44:4	3.29E-03
His	2.61E-06	PC aa C36:2	4.74E-05	PC ae C44:5	1.61E-03
Ile	2.89E-03	PC aa C36:3	2.93E-05	PC ae C44:6	1.94E-03

Metabolite	p-value (Wilcoxon)	Metabolite	p-value (Wilcoxon)	Metabolite	p-value (Wilcoxon)
Leu	8.24E-04	PC aa C36:4	5.43E-05	SM (OH) C14:1	5.01E-04
Lys	1.23E-02	PC aa C36:5	1.88E-04	SM (OH) C16:1	2.82E-04
Met	1.46E-02	PC aa C36:6	8.54E-03	SM (OH) C22:1	5.42E-03
Orn	1.38E-07	PC aa C38:0	5.98E-04	SM (OH) C22:2	1.48E-03
Phe	6.28E-08	PC aa C38:1*	1.29E-01	SM (OH) C24:1	4.79E-03
Pro	1.68E-06	PC aa C38:3	4.43E-05	SM C16:0	1.46E-04
Ser	2.80E-08	PC aa C38:4	8.68E-05	SM C16:1	2.57E-04
Thr	2.07E-03	PC aa C38:5	6.01E-05	SM C18:0	1.20E-04
Trp	3.31E-07	PC aa C38:6	4.91E-05	SM C18:1	4.59E-04
Tyr	9.58E-05	PC aa C40:1	4.70E-02	SM C20:2	2.45E-02
Val	8.42E-03	PC aa C40:2	3.55E-03	SM C24:0	3.46E-03
		PC aa C40:3	1.54E-02	SM C24:1	6.73E-04
		PC aa C40:4	3.37E-03	SM C26:0*	5.25E-01
		PC aa C40:5	9.90E-05	SM C26:1	2.12E-01
		PC aa C40:6	5.43E-05	Hexose	6.45E-01
		PC aa C42:0	1.05E-02		
		PC aa C42:1	2.98E-01		
		PC aa C42:2	1.07E-01		
		PC aa C42:4	6.30E-02		
		PC aa C42:5	4.70E-02		
		PC aa C42:6	6.53E-02		

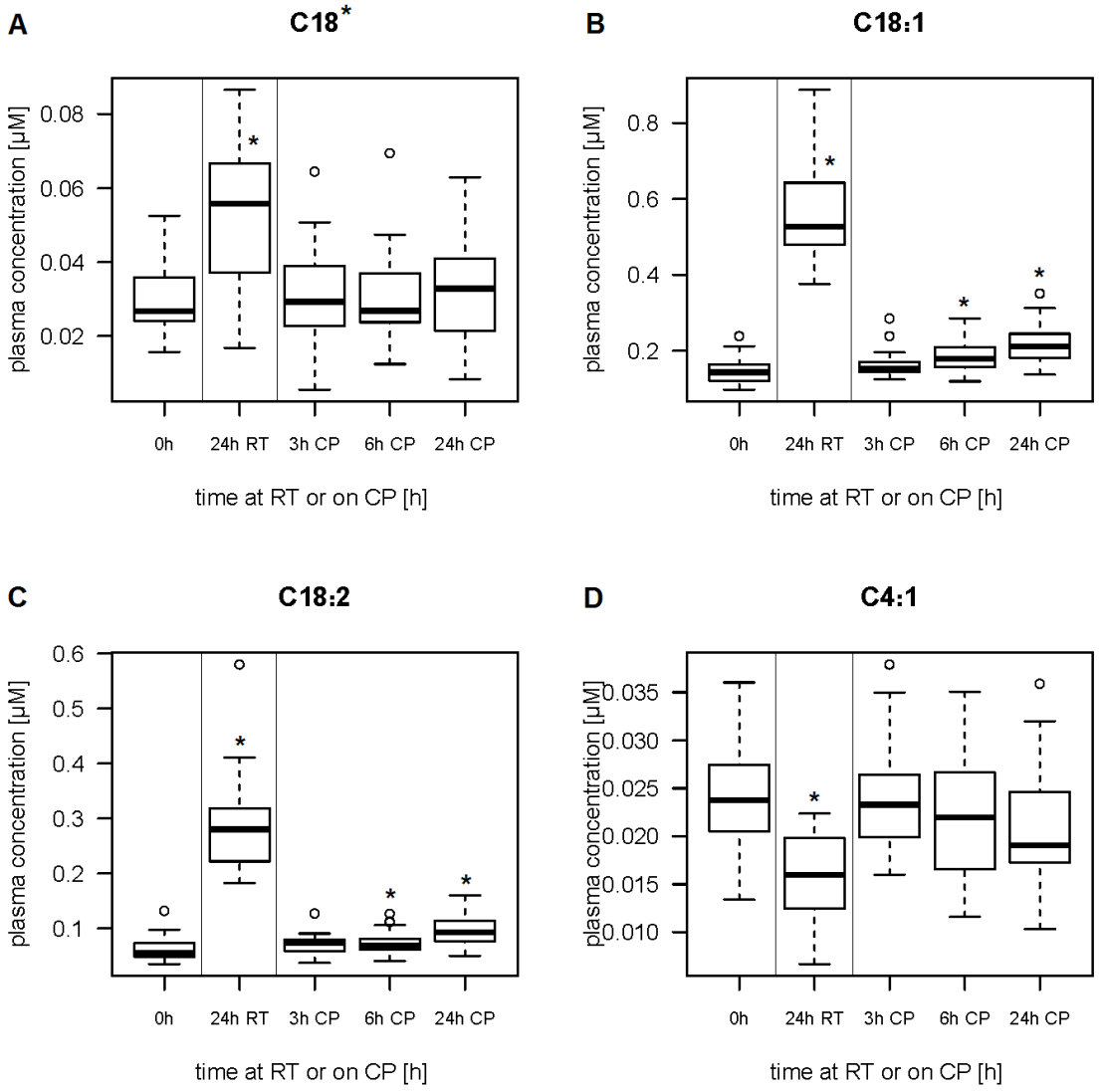
SUPPLEMENTARY TABLE 5. Impact of simulated shipment on metabolite ratios in plasma samples. Metabolite ratios showed significant changes in plasma samples that were stored at RT for 24h compared with baseline (Wilcoxon signed rank test, p < 4.03E-06, p-gain > 1580).

Metabolite ratio		
C2/PC aa C30:0	PC aa C32:0/PC ae C44:5	PC ae C34:1/PC ae C36:5
C2/PC ae C36:2	PC aa C34:1/PC ae C36:5	PC ae C34:1/PC ae C38:6
Gly/PC aa C34:2	PC aa C34:1/PC ae C38:4	PC ae C34:1/PC ae C44:5
Gly/PC ae C34:1	PC aa C34:1/PC ae C38:6	PC ae C34:2/PC ae C38:3
Val/PC aa C32:0	PC aa C34:1/PC ae C40:6	PC ae C34:2/PC ae C38:5
Val/PC aa C34:2	PC aa C34:1/PC ae C44:5	PC ae C36:2/PC ae C36:5
Val/PC aa C36:2	PC aa C34:2/PC ae C34:3	PC ae C36:2/PC ae C40:6
Val/PC aa C36:5	PC aa C34:2/PC ae C36:5	PC aa C34:1/PC aa C38:3
Val/PC ae C34:1	PC aa C34:2/PC ae C38:6	PC aa C34:1/PC aa C38:4
Val/PC ae C34:2	PC aa C34:4/PC ae C34:3	PC aa C34:1/PC aa C38:5
Val/PC ae C36:2	PC aa C34:4/PC ae C36:5	PC aa C34:1/PC aa C38:6
SM C16:0/PC aa C34:1	PC aa C34:4/PC ae C38:3	PC aa C34:2/PC aa C38:3
SM C16:0/PC aa C34:2	PC aa C34:4/PC ae C38:6	PC aa C34:2/PC aa C38:6
SM C16:0/PC aa C36:2	PC aa C36:1/PC ae C40:6	PC aa C34:2/PC aa C42:6
SM C16:0/PC ae C36:2	PC aa C36:2/PC ae C36:5	PC aa C36:1/PC aa C38:3
SM C18:0/PC aa C30:0	PC aa C36:2/PC ae C38:4	PC aa C36:1/PC aa C38:6
SM C18:0/PC aa C32:0	PC aa C36:2/PC ae C38:6	PC aa C36:2/PC aa C38:3
SM C18:0/PC aa C34:1	PC aa C36:2/PC ae C44:5	PC aa C36:2/PC aa C38:5
SM C18:0/PC aa C34:2	PC aa C36:3/PC ae C36:2	PC aa C36:2/PC aa C38:6
SM C18:0/PC aa C36:2	PC aa C36:5/PC ae C36:5	PC aa C36:4/PC aa C38:3
SM C18:0/PC aa C36:5	PC aa C36:5/PC ae C38:6	PC aa C36:5/PC aa C38:3
SM C18:0/PC ae C36:2	PC aa C36:5/PC ae C40:6	PC aa C36:5/PC aa C38:5
SM C24:1/PC ae C36:2	PC aa C38:3/PC ae C34:1	
	PC aa C38:3/PC ae C34:2	
	PC aa C38:3/PC ae C36:2	
	PC aa C38:4/PC ae C36:2	
	PC aa C38:5/PC ae C36:2	
	PC aa C38:6/PC ae C36:2	
	PC aa C40:6/PC ae C36:2	



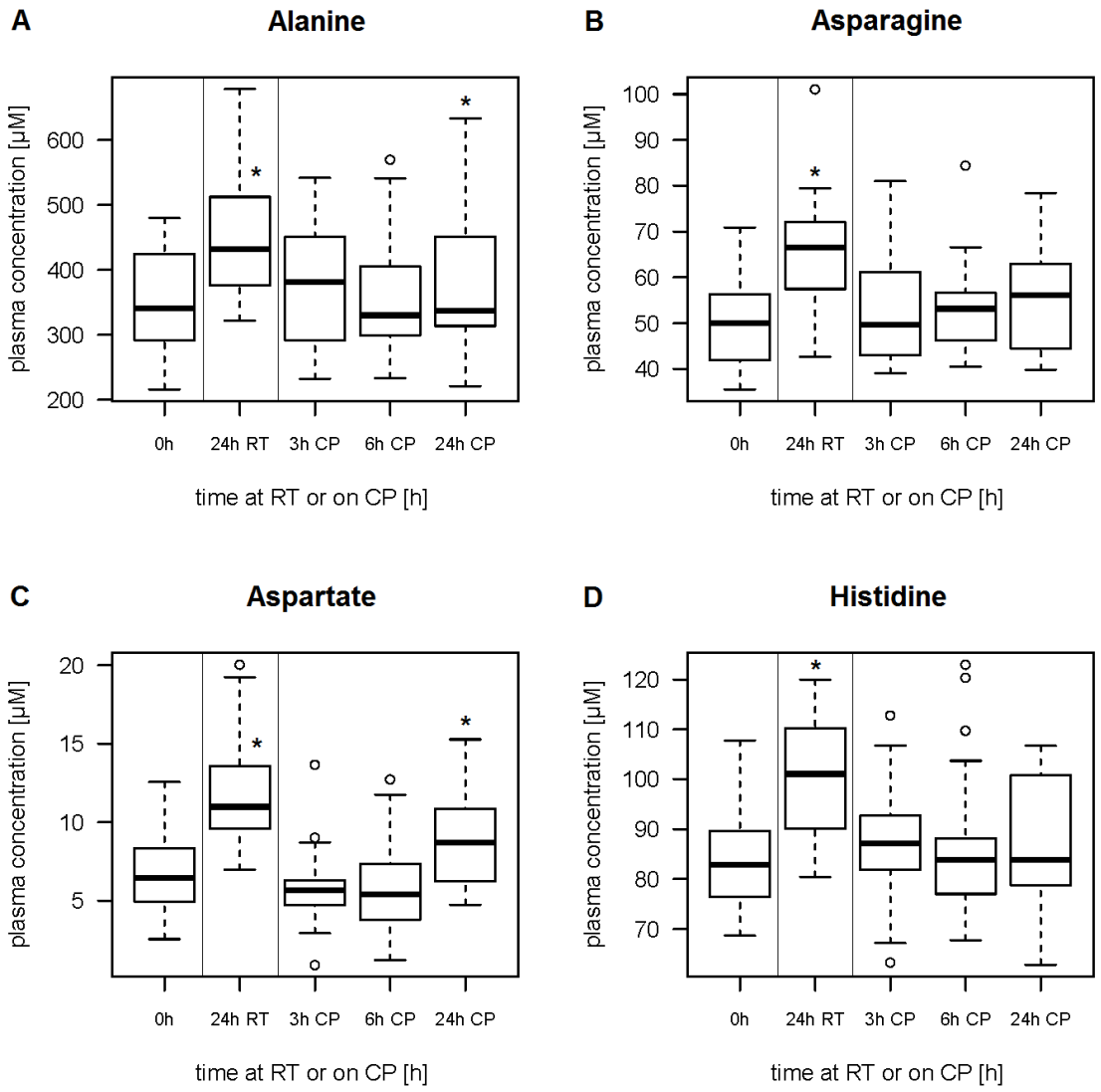
SUPPLEMENTARY FIGURE 1. Changes in metabolite concentration during simulated transport of plasma samples.

(A) C10:2, (B) C14*, (C) C14:1-OH*, and (D) C16. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



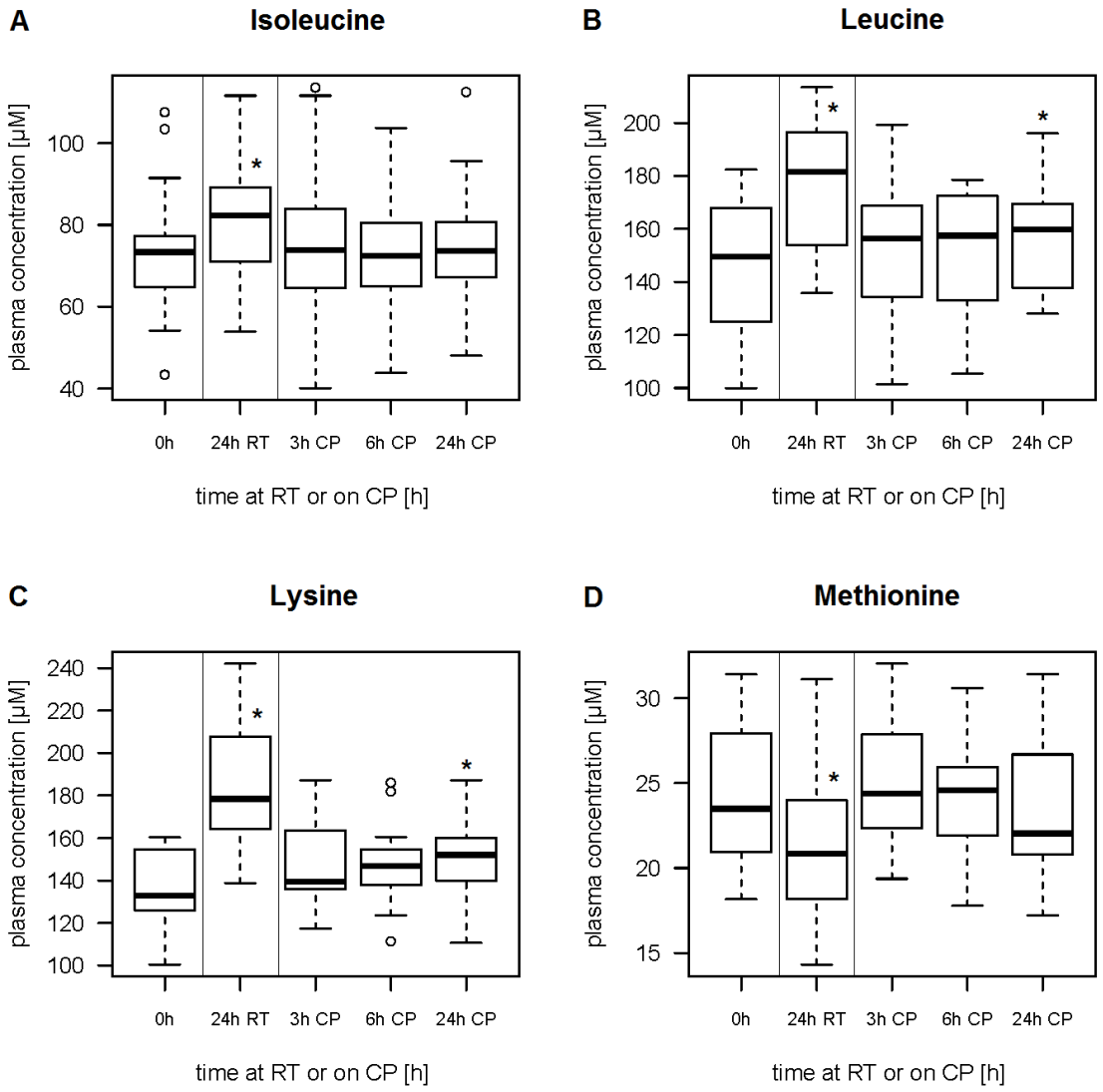
SUPPLEMENTARY FIGURE 2. Changes in metabolite concentration during simulated transport of plasma samples.

(A) C18*, (B) C18:1, (C) C18:2, and (D) C4:1. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



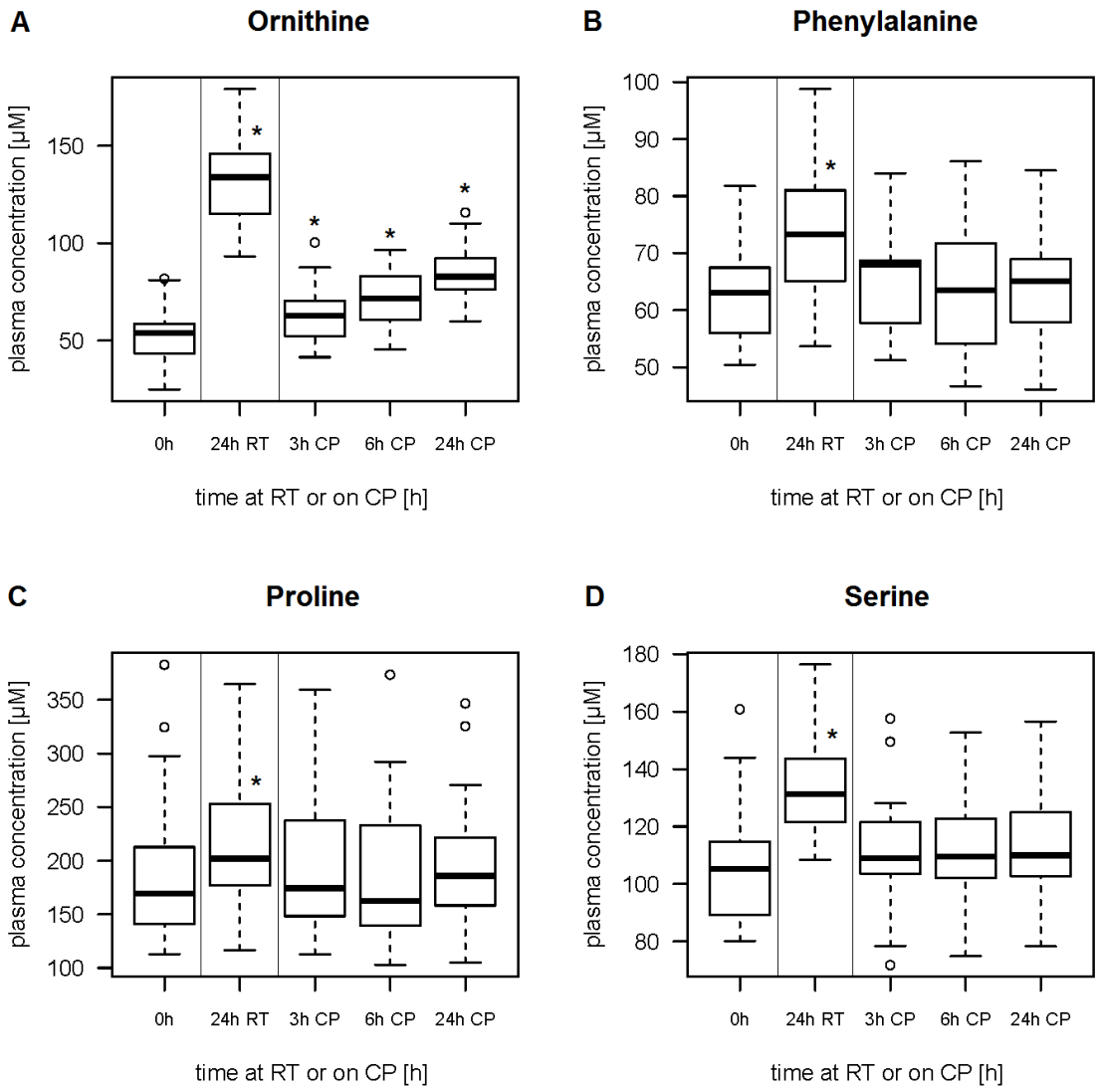
SUPPLEMENTARY FIGURE 3. Changes in metabolite concentration during simulated transport of plasma samples.

(A) Ala, (B) Asn, (C) Asp, and (D) His. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



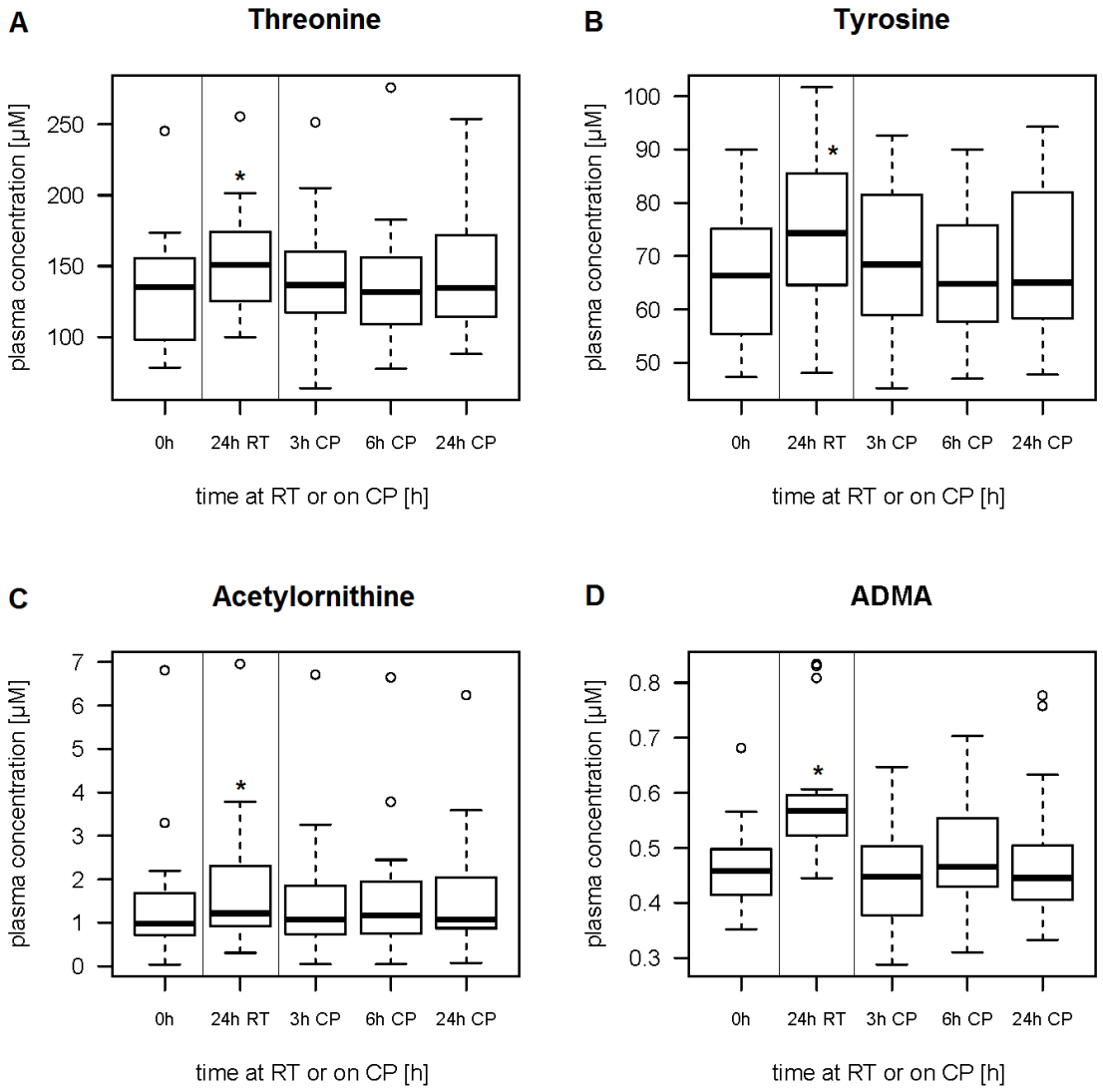
SUPPLEMENTARY FIGURE 4. Changes in metabolite concentration during simulated transport of plasma samples.

(A) Ile, (B) Leu, (C) Lys, and (D) Met. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



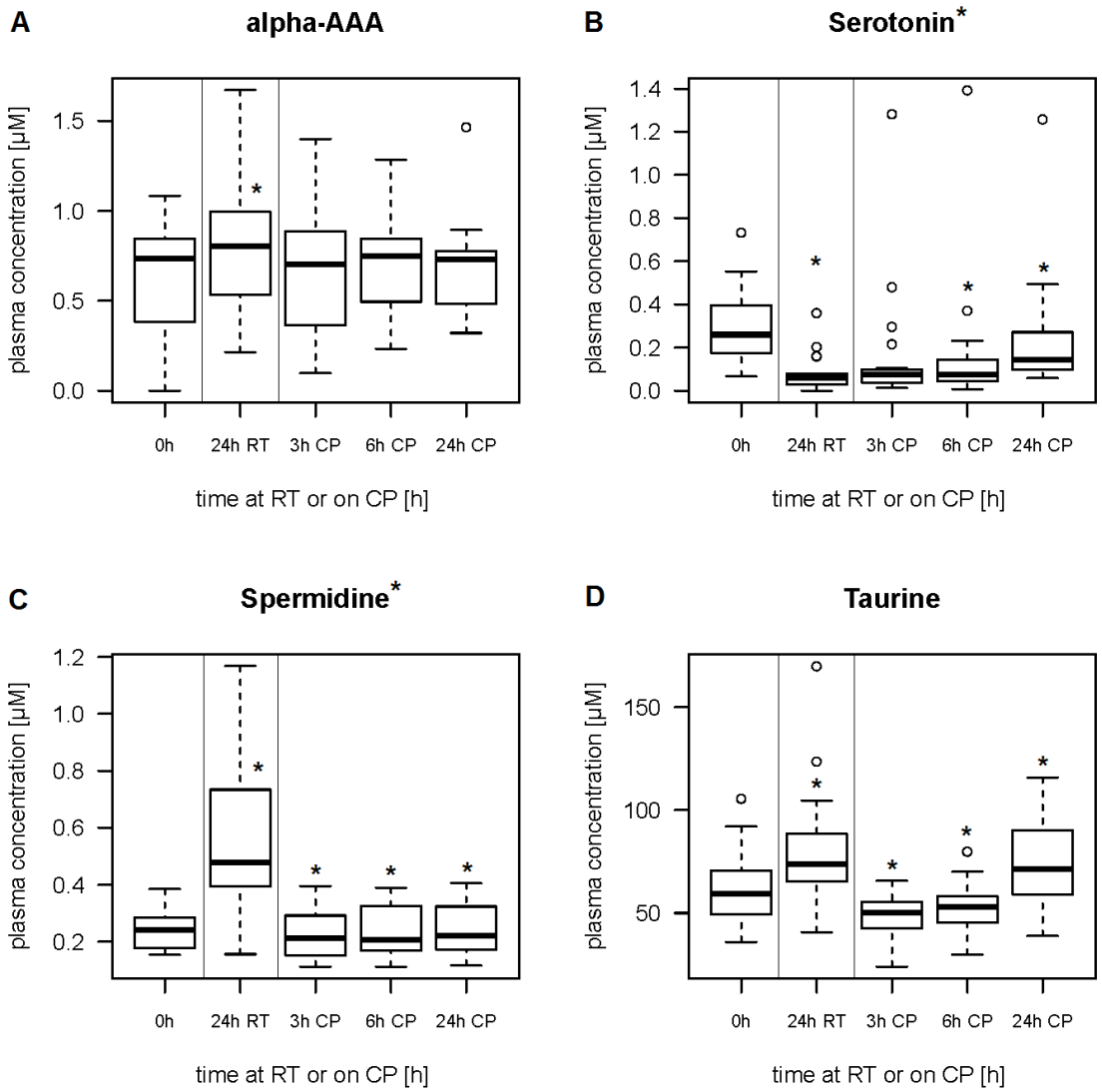
SUPPLEMENTARY FIGURE 5. Changes in metabolite concentration during simulated transport of plasma samples.

(A) Orn, (B) Phe, (C) Pro, and (D) Ser. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



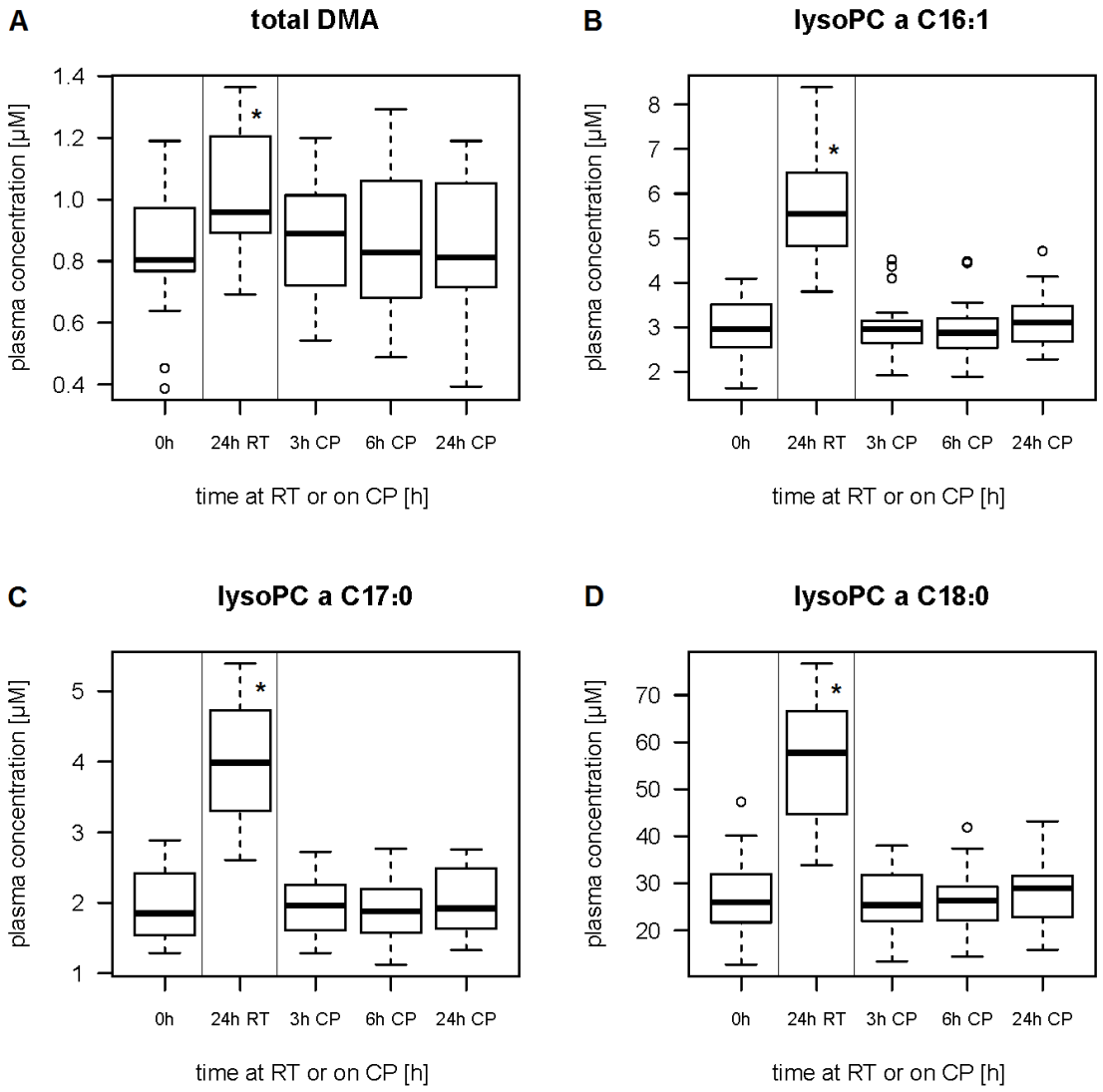
SUPPLEMENTARY FIGURE 6. Changes in metabolite concentration during simulated transport of plasma samples.

(A) Thr, (B) Tyr, (C) Acetylornithine, and (D) ADMA. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



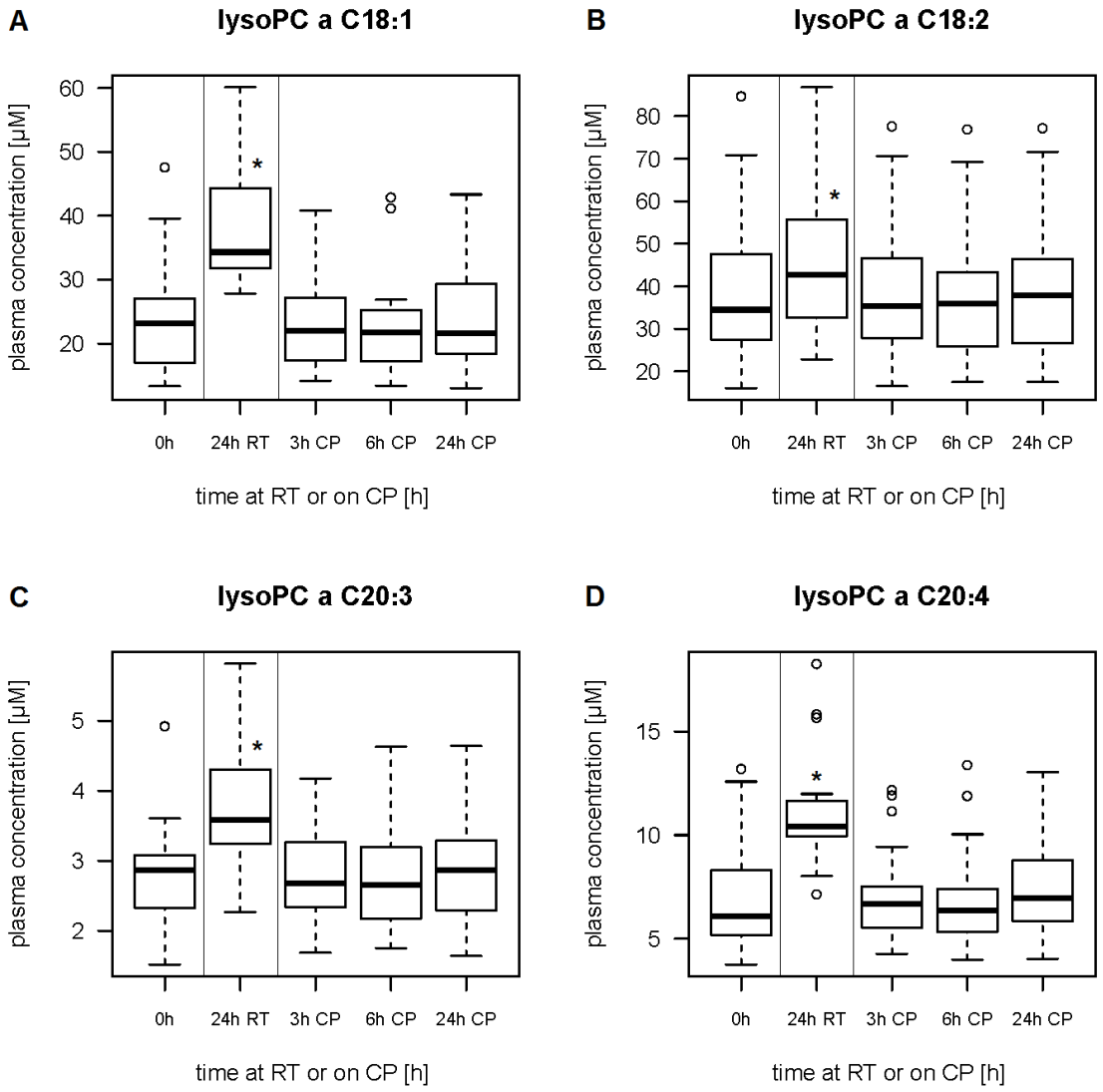
SUPPLEMENTARY FIGURE 7. Changes in metabolite concentration during simulated transport of plasma samples.

(A) alpha-AAA, (B) Serotonin*, (C) Spermidine*, and (D) Taurine. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



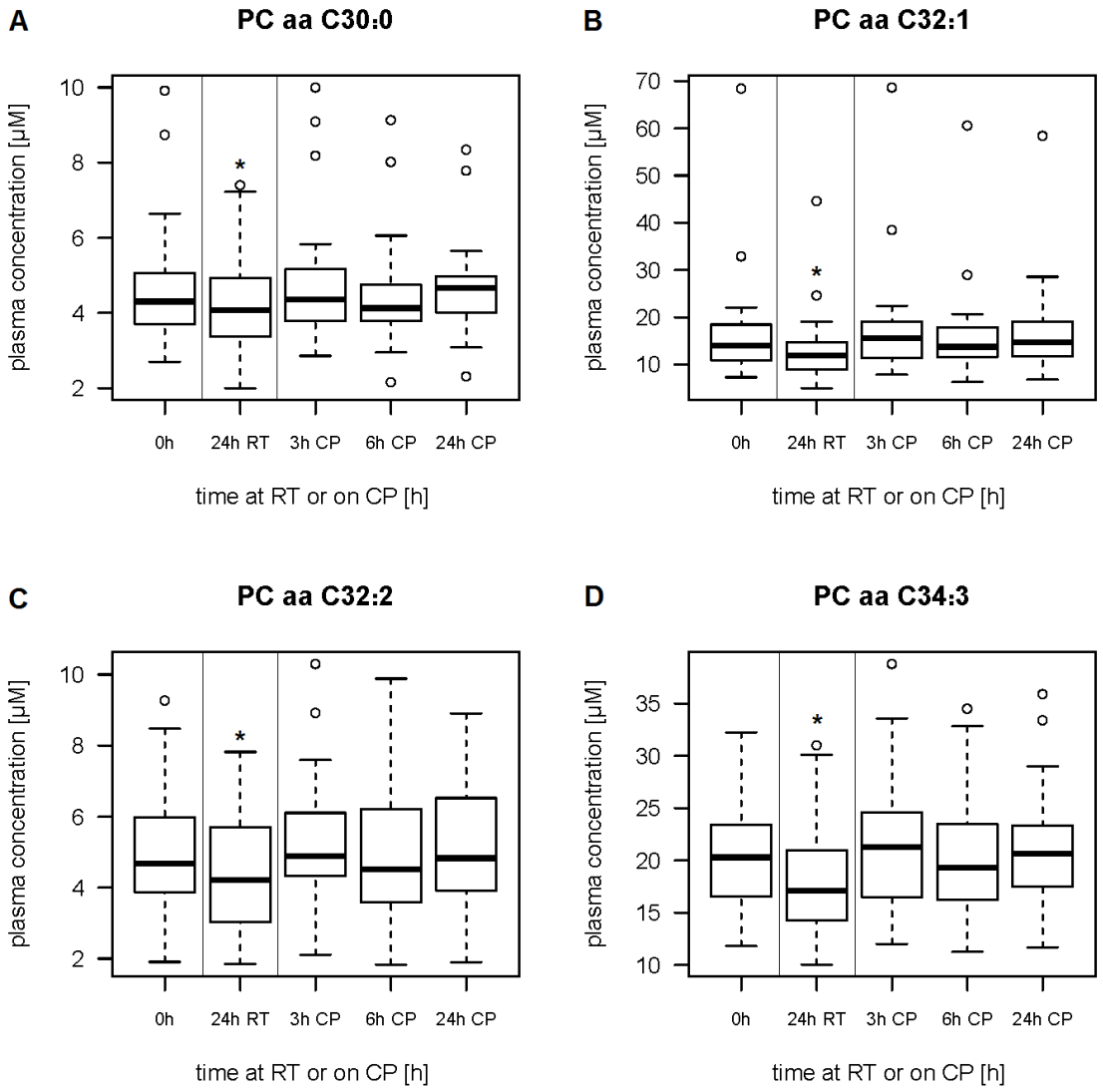
SUPPLEMENTARY FIGURE 8. Changes in metabolite concentration during simulated transport of plasma samples.

(A) total DMA, (B) lysoPC a C16:1, (C) lysoPC a C17:0, and (D) lysoPC a C18:0. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



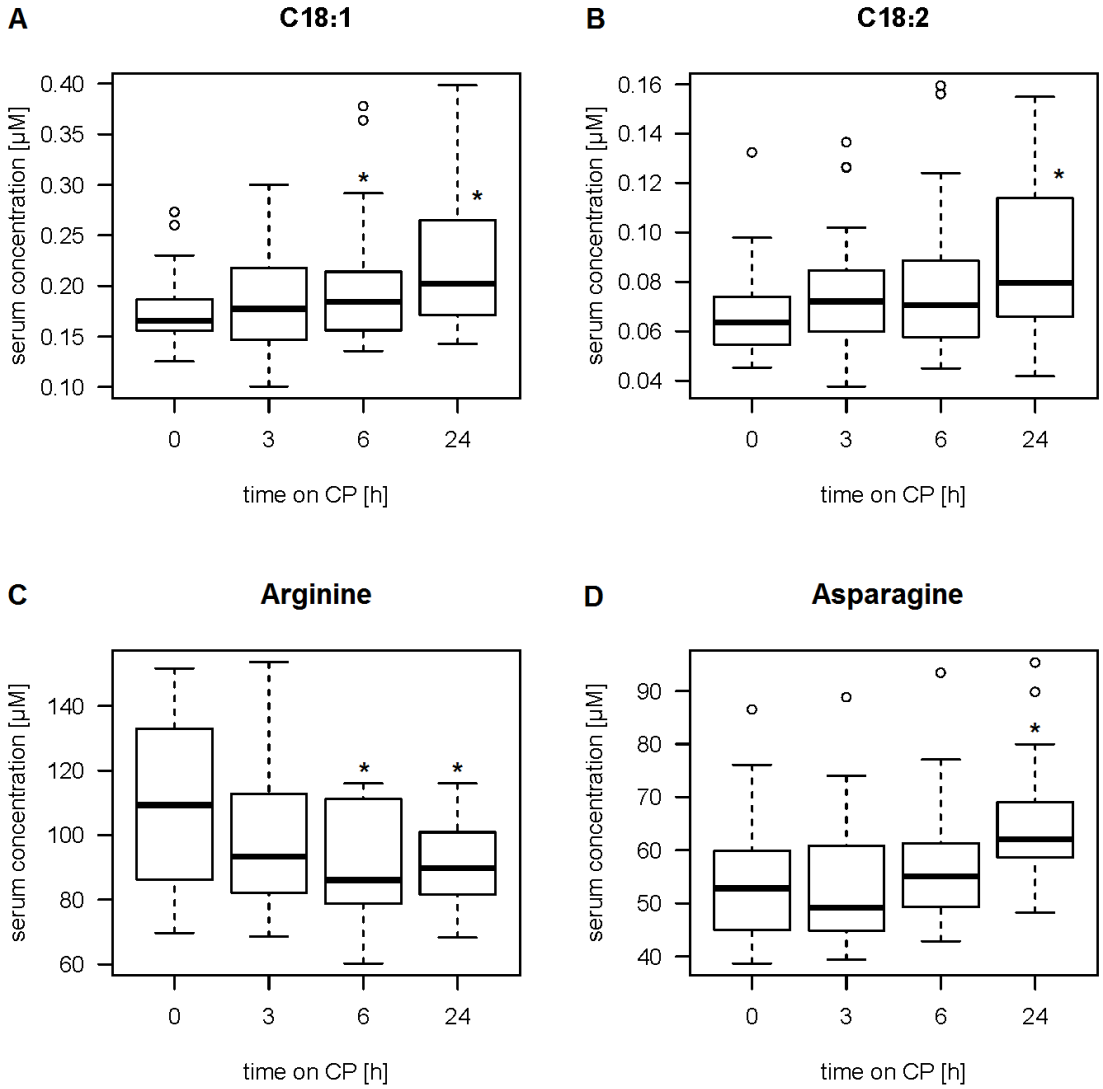
SUPPLEMENTARY FIGURE 9. Changes in metabolite concentration during simulated transport of plasma samples.

(A) lysoPC a C18:1, (B) lysoPC a C18:2, (C) lysoPC a C20:3, and (D) lysoPC a C20:4. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



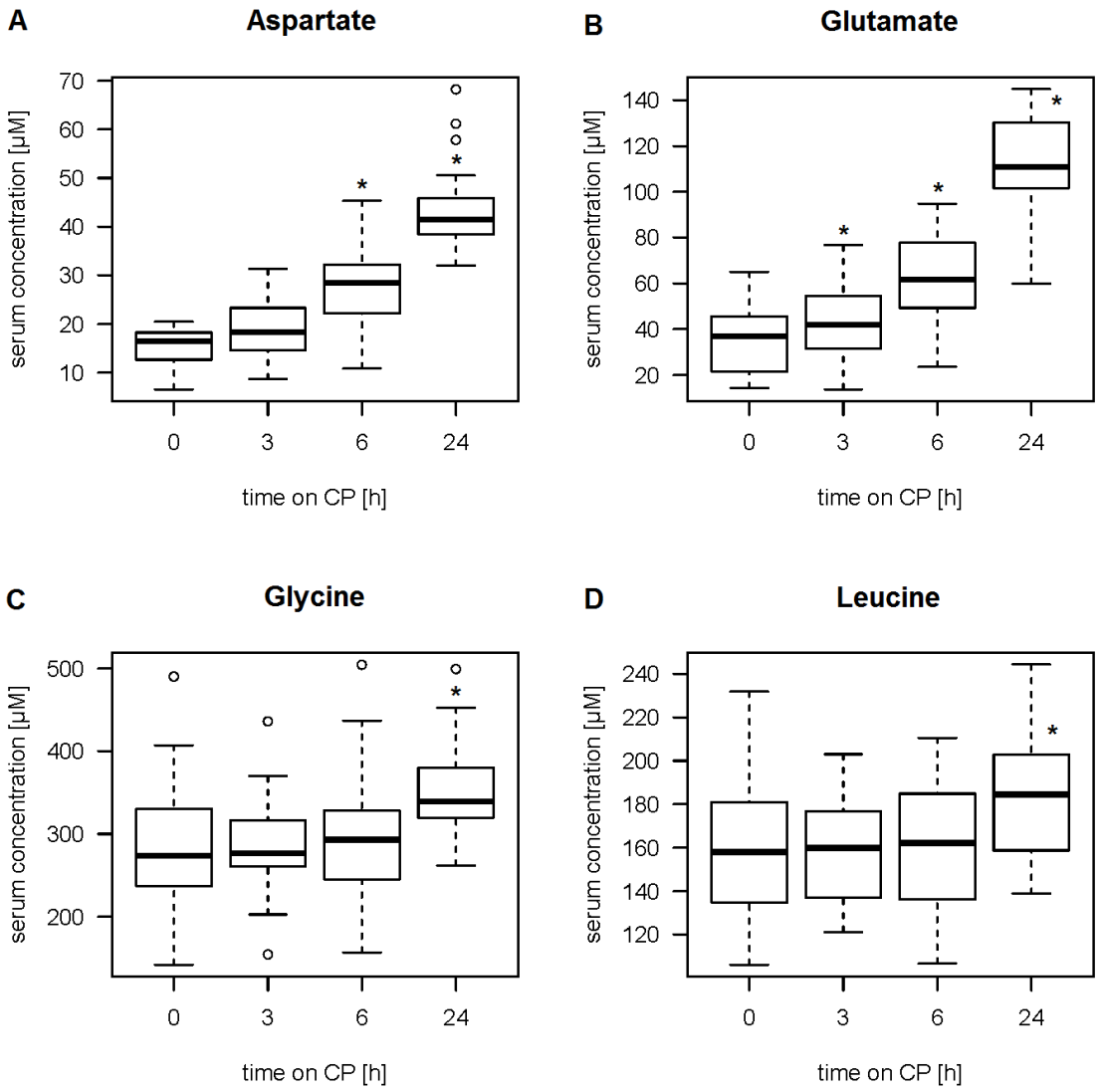
SUPPLEMENTARY FIGURE 10. Changes in metabolite concentration during simulated transport of plasma samples.

(A) PC aa C30:0, (B) PC aa C32:1, (C) PC aa C32:2, and (D) PC aa C34:3. Vertical lines in the boxplots indicate that time points on the x-axis are not sorted chronologically. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



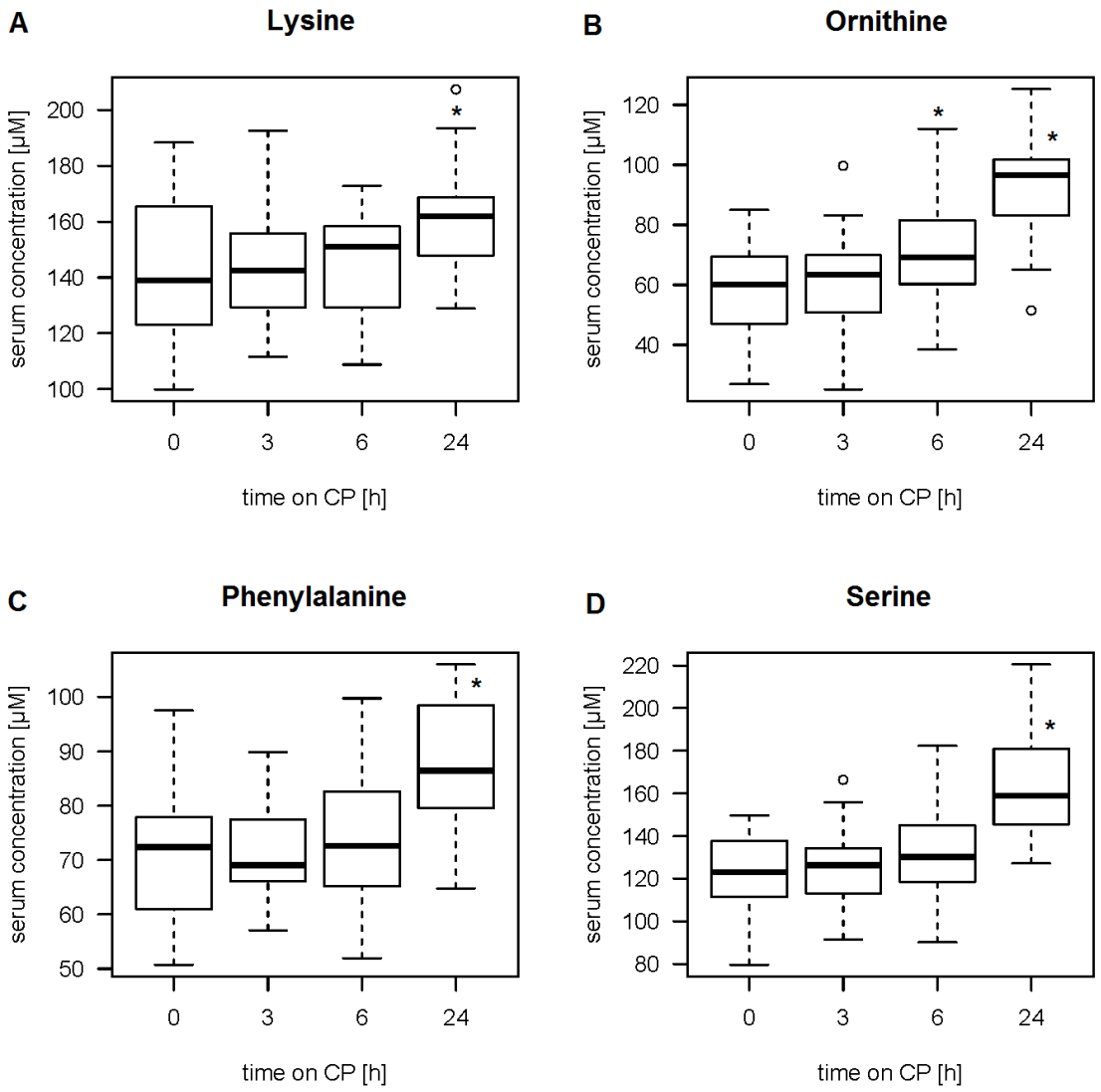
SUPPLEMENTARY FIGURE 11. Changes in metabolite concentration during simulated transport of serum samples.

(A) C18:1, (B) C18:2, (C) Arg, and (D) Asn. Asterisks in boxplot indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



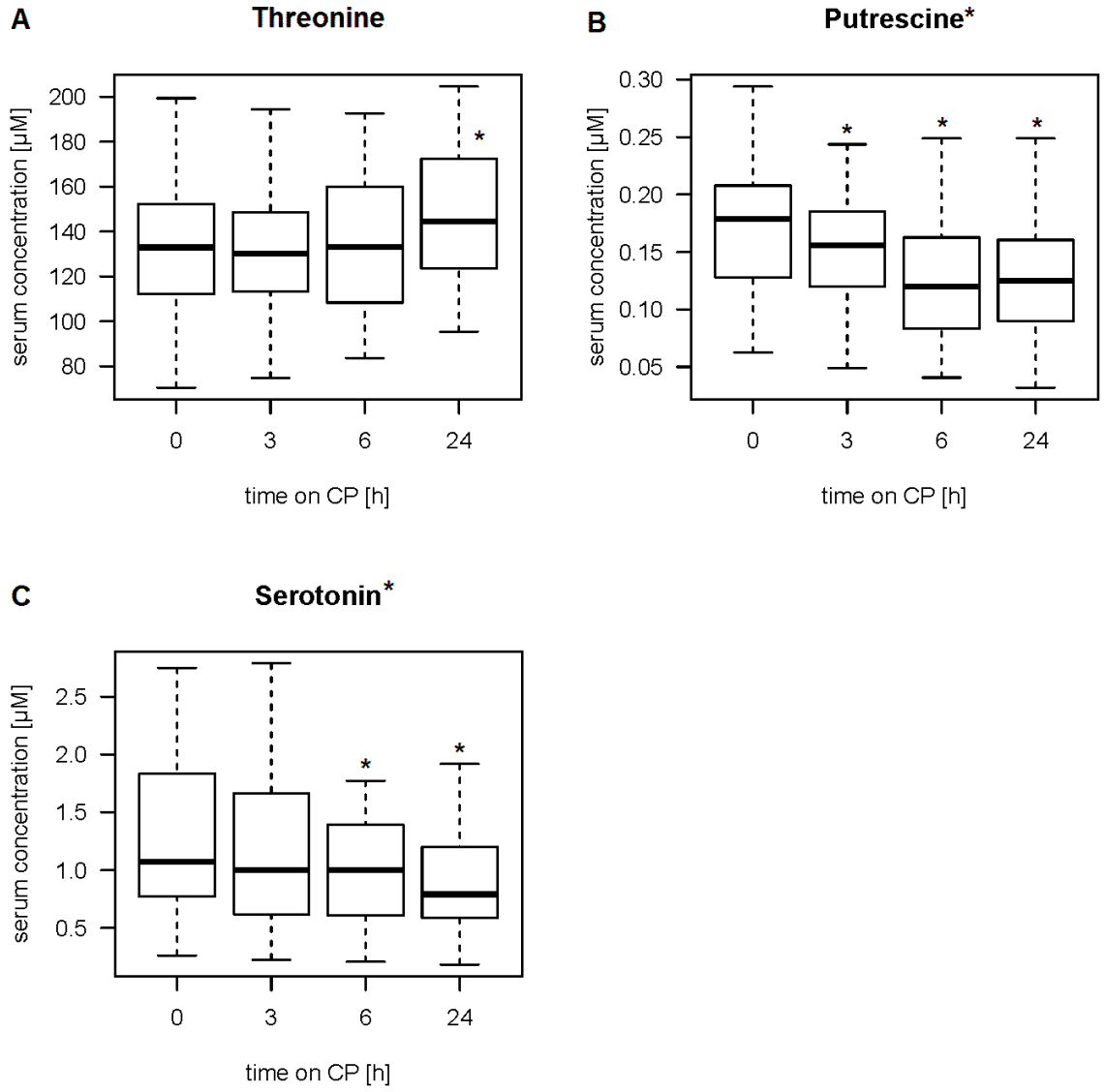
SUPPLEMENTARY FIGURE 12. Changes in metabolite concentration during simulated transport of serum samples.

(A) Asp, (B) Glu, (C) Gly, and (D) Leu. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



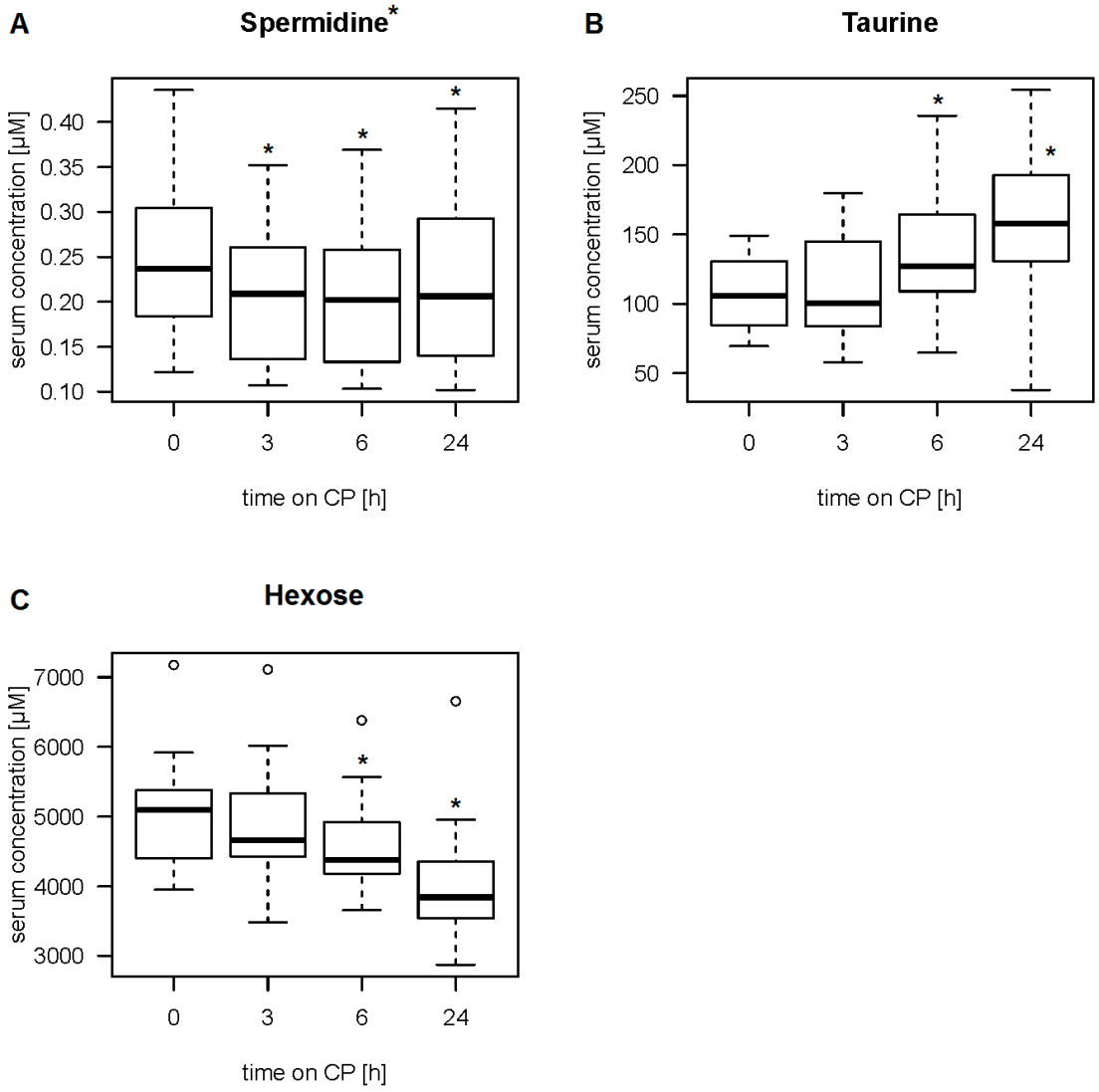
SUPPLEMENTARY FIGURE 13. Changes in metabolite concentration simulated transport of serum samples.

(A) Lys, (B) Orn, (C) Phe, and (D) Ser. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



SUPPLEMENTARY FIGURE 14. Changes in metabolite concentration during simulated transport of serum samples.

(A) Thr, (B) Putrescine*, and (C) Serotonin*. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).



SUPPLEMENTARY FIGURE 15. Changes in metabolite concentration simulated transport of serum samples.

(A) Spermidine*, (B) Taurine, and (C) Hexose. Asterisks in boxplots indicate significant difference in concentration compared with baseline (0h). (Tested in a pairwise manner against the reference (0h) with Wilcoxon signed-rank tests, significance level $p < 0.01$).

PUBLICATIONS

Breier M, Wahl S, Prehn C, Ferrari U, Sacco V, Weise M, Grallert H, Adamski J & Lechner A. Metabolic changes in patients with newly diagnosed type 2 diabetes induced by single dose and short-term metformin intake *(submitted)*.

Breier M, Wahl S, Prehn C, Fugmann M, Ferrari U, Weise M, Banning F, Seissler J, Grallert H, Adamski J & Lechner A. Targeted Metabolomics Identifies Reliable and Stable Metabolites in Human Serum and Plasma Samples. *PLoS One* **9(2)**: e89728. doi:10.1371/journal.pone.0089728, 2014.

Albrecht E, Waldenberger M, Krumsiek J, Evans A, Jeratsch U, **Breier M**, Adamski J, Koenig W, Zeilinger S, Fuchs C, Klopp N, Theis F, Wichmann HE, Suhre K, Illig T, Strauch K, Peters A, Gieger C, Kastenmüller G, Doering A & Meisinger C. Metabolite profiling reveals new insights into the regulation of serum urate in humans. *Metabolomics* **10** 141-151, 2014.

Wahl S, Holzapfel C, Yu Z, **Breier M**, Kondofersky I, Fuchs C, Singmann P, Prehn C, Adamski J, Grallert H, Illig T, Wang-Sattler R & Reinehr T. Metabolomics reveals determinants of overweight reduction during lifestyle intervention in obese children. *Metabolomics* **9(6)** 1157-1167, 2013.

ACKNOWLEDGEMENTS

Mein besonderer Dank gilt meinem Doktorvater Herrn Prof. Dr. Jerzy Adamski für die anregenden Diskussionen und sein stetes Interesse am Fortgang dieser Arbeit.

Frau Prof. Dr. Hannelore Daniel möchte ich recht herzlich danken, dass sie sich bereit erklärt hat das Zweitgutachten für diese Arbeit zu erstellen.

Ein großer Dank gilt meinem Betreuer Dr. Harald Grallert für die Möglichkeit diese Arbeit durchführen zu können und für die gute wissenschaftliche Betreuung.

Meinen Kooperationspartnern der Arbeitsgruppe Lechner (Diabetes Research Group, Klinikum der Universität München) und Prof. Torben Hansen (Universität Kopenhagen) danke ich für die Zusammenarbeit und die Bereitstellung der Studiendaten. Außerdem gilt mein Dank den Mitarbeitern des Instituts für Experimentelle Genetik, insbesondere Dr. Cornelia Prehn, Dr. Anna Artati, Julia Scarpa, Andrea Neftzger und Katharina Faschinger für die targeted Metabolomics Messungen und Mark Haid für die Zusammenarbeit im IMI-DIRECT Projekt.

Bei meinen Kolleginnen und Kollegen möchte ich mich herzlich für ihre Hilfsbereitschaft und das angenehme Arbeitsklima bedanken, insbesondere bei Simone für ihre guten und wertvollen Ratschläge im Bereich Statistik und bei Paula und Rory für das Korrekturlesen meiner Arbeit.

Zuletzt gilt mein Dank meinen Eltern sowie meinem Verlobten und meinen Freunden, die mich stets unterstützt haben.