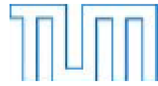

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Metabolomic analysis of antidiabetic drug action

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Abstract

To examine the response of the metabolic phenotype to diabetes and antidiabetic medication is of great importance in drug discovery and patient therapy, since it allows the determination of new drug targets and improves the understanding of the underling drug mechanisms. Pathological processes as well as action of a drug, affect organs in a site specific manner. Therefore, technologies offering the detection and monitoring of the metabolic response from specific body regions or organs are required. Here mass spectrometry based metabolomics is the tool of choice as it allows the simultaneously and sensitive tracing of numerous metabolites, and their change in response to pharmacological interventions.

In this work, metabolomics were used to study antidiabetic drug action in peripheral organs (testis, lung, adrenal gland, cerebellum) and on the systemic level (plasma). The obtained data can extend the present knowledge about standard and novel therapeutic intervention in the field of diabetes care. Three different antidiabetic therapies were applied to a diabetic mouse model. Two single drugs (metformin, SGLT2-inhibitor), and a combination of both drugs were administered. Each treatment was administered as one single dose, or as repeated daily administration over 14 days. Subsequently, the metaboloic response to the acute and sub-chronic antidiabetic intervention was investigated.

All measured metabolites and treatment groups underwent strict statistical evaluation, using a linear model and applying outlier control and correction for multiple testing to exclude artifacts. The utilization of ratios between metabolite concentrations dramatically reduces the noise in the data set. Furthermore, metabolite ratios enable the detection of correlations between metabolites, which were not detectable for the single metabolites. This allows the investigation of biochemical pathways.

During the acute antidiabetic intervention, SGLT2-inhibitor treatment had the greatest impact on metabolism. Most alterations were found for amino acids and acylcarnitines. These metabolic alterations were similar to those observed during fasting. It was hypothesized that a long-term intervention with SGLT2-inhibitor can mimic the effects of fasting and therefore led to a normalization of diabetes related factors.

During the sub-chronic intervention, metformin was mainly the cause of action for the observed metabolic alteration. However, metformin treatment alone failed to normalize blood glucose levels. This was only reached during combination treatment with both antidiabetic drugs. The metabolomics patterns for metformin and combination treatment are similar to each other. However, the combination

treatment results in specific response of several metabolite levels and biochemical pathways, which can be responsible for the observed combinatory effect on blood glucose normalization. On the systemic level (in plasma), citrulline was the most effected single metabolite. Citrulline is a marker for vascular health, linking improvement in cardiovascular status to the antidiabetic intervention. Furthermore, the diabetic biomarker, amino adipic acid was found altered by combination therapy. Additionally, the results of the metabolite classes phosphatidylocholines and lysophosphatidylcholines reflect alteration in membrane lipid turnover, possible caused by the action of phospholipase A2 or related enzymes. Finally, systemic hydroxy sphingomyelin levels were found to be altered by metformin and combination treatment. Site specific response to sub-chronic combination treatment was observed for long-chain hydroxylated acylcarnitines in the lung. Additionally, the histamine and carnosine concentration were altered in cerebellum. The observed alterations, in part explain the observed combinatory drug effect, and are possible targets for new antidiabetic drugs.

The treatment of common, multi-factorial diseases such as the metabolic syndrome requires multi-component therapeutics. The pharmacokinetic interaction of different drug components is one of the challenges in this approach. Additionally, pharmacokinetics of drugs in tissues depends on organ specific mechanisms, and can result in site specific response to the drug. Thus, a LC-MS/MS based quantification method for metformin was established, which allows for the detection of metformin in mouse tissue and plasma. The pharmacokinetic data obtained with this assay indicates an influence of SGLT2-inhibitor action on metformin pharmacokinetics in tissues and on the systemic level.

Zusammenfassung

Die Untersuchung der Antwort des metabolischen Phänotyps auf Diabetes und die antidiabetische Behandlung ist von großer Bedeutung in der Wirkstoffforschung als auch für die Therapie von Patienten. Sie ermöglicht die Ermittlung neuer Wirkstoffziele und verbessert das Verständnis der zugrundeliegenden Wirkmechanismen. Sowohl pathologische Prozesse, als auch die Wirkung eines Arzneimittels, beeinflussen Organe und Gewebe in einer jeweils spezifischen Weise. Aus diesem Grund werden Technologien benötigt, welche den Nachweis und die Beobachtung metabolischer Reaktionen in einzelnen Organen ermöglichen. Eine der am besten geeignetsten Techniken hierfür ist die auf massenspektrometrischen Methoden basierend Metabolomik. Sie ermöglicht die sensitive und simultane Verfolgung der Veränderung einer Vielzahl von Metaboliten in Reaktion auf pharmakologische Interventionen.
In dieser Arbeit wurden Analysemethoden aus der Metabolomik verwendet, um die Wirkung von Antidiabetika in peripheren Organen (Hoden, Lunge, Nebenniere, Cerebellum) und auf der systemischen Ebene (Plasma) zu studieren. Die gewonnen Daten können das aktuelle Wissen über die Wirkung von neuen Medikamenten und Standardpräparaten in der Diabetesbehandlung erweitern. Drei verschiedene antidiabetische Therapien wurden auf ein diabetisches Mausmodell angewandt. Zwei Therapien bestanden aus einzelnen Medikamenten (Metformin und ein SGLT2-Inhibitor). Als dritte Therapie wurde eine Kombination der beiden Medikamente verabreicht. Jede Behandlung wurde entweder als Einzelgabe oder als täglich wiederholte Gabe über 14 Tage verabreicht. Anschließend wurde die metabolische Reaktion auf die akute und subchronische Behandlung untersucht.

Um Artefakte auszuschließen durchliefen alle gemessenen Metaboliten aus den Behandlungsgruppen eine strenge statistische Auswertung. Die Verwendung von Verhältnissen zwischen Metabolitenkonzentrationen reduziert drastisch das statistische Rauschen im Datensatz. Zudem ermöglichte die Betrachtung von Verhältnissen zwischen Metabolitenkonzentrationen die Entdeckung von Korrelationen zwischen den Metaboliten, welche für den einzelnen Metabolit nicht nachweisbar waren. Damit war auch die Untersuchung der zugrundeliegenden biochemischen Wege besser möglich.

Während der akuten Gabe der Antidiabetika hatte der SGLT2-Inhibitor den größten Einfluss auf den Stoffwechsel. Die meisten der beobachteten Änderungen traten dabei bei den Aminosäuren und Acylcarnitinen auf. Die in diesen beiden Stoffklassen beobachteten Veränderungen waren dabei ähnlich zu denen die während des Fastens auftreten. Es wurde die Hypothese aufgestellt, dass eine

langfristige Gabe des SGLT2-Inhibitors zu ähnlichen Effekten führt wie sie während des Fastens beobachtete werden. Dies könnte dann zu einer Normalisierung von metabolischen Faktoren führen die in Zusammenhang mit Diabetes stehen.

Während der subchronischen Intervention war Metformin die Hauptursache der beobachteten metabolischen Veränderungen. Allerdings, gelang es nicht den Blutzuckerspiegel dauerhaft nur durch Metformin zu kontrollieren. Dies wurde nur durch die Kombinationsbehandlung mit beiden Medikamenten erreicht. Die während der Metformin- und Kombinationsbehandlung beobachteten metabolischen Muster waren sehr ähnlich. Die Kombinationsbehandlung führte jedoch zu spezifischen Änderungen in den Konzentrationen mehrerer Metabolite und einzelner Stoffwechselwege. Diese spezifischen Änderungen könnten einen Anteil an der beobachteten Normalisierung der Blutglukose durch die Kombinationsbehandlung habe. Auf systemischer Ebene (im Plasma) war Citrullin der am stärksten veränderte Metabolit. Citrullin ist ein Marker für vaskuläre Gesundheit und zeigt eine Verbindung zwischen Herz-Kreislaufstatus und antidiabetische Therapie. Des Weiteren wurden Änderungen der Plasmakonzentration der Aminoadipinsäure festgestellt, welche als Biomarker für Diabetes postuliert ist. Die Ergebnisse der Metabolitenklassen Phosphatidylcholine und Lysophosphatidylcholine deuten auf Änderungen in der Membranzusammensetzung hin. Diese Ergebnisse reflektieren Änderungen in der Aktivität der Phospholipase A2 oder verwandter Enzyme. Schließlich wurden im Plasma Konzentrationsänderungen der Hydroxy-Sphingomyeline gefunden, die sowohl in der Metformin als auch in der Kombinationsbehandlung auftreten. Organspezifische Reaktionen auf die subchronische Kombinationsbehandlung konnten in der Lunge beobachtet werden. Hier führte die Behandlung zu Änderungen in der Konzentration der langkettigen hydroxylierten Acylcarnitine. Im Cerebellum konnten Änderungen im Polyaminehaushalt festgestellt werden. Hier änderten sich die Konzentrationen von Histamin und Carnosin. Die beobachteten Veränderungen erklären teilweise die verstärkte Wirkung der kombinatorischen Therapie auf den Blutzuckerspiegel. Damit sind sie mögliche Ziele für neue Antidiabetika.

Die Behandlung allgemeiner, multifaktorieller Krankheiten wie des metabolischen Syndroms erfordert den Einsatz von Arzneimittelkombinationen. Die pharmakokinetische Interaktion verschiedener Wirkstoffkomponenten ist eine der Herausforderungen beim Einsatz von Kombinationspräparaten. Darüber hinaus hängt die Pharmakokinetik von Arzneimitteln in Geweben von organspezifischen Mechanismen ab. Dies kann zu organspezifischen Reaktionen auf das Medikament führen. Aus diesem Grund wurde ein LC -MS / MS- basiertes Detektionsverfahren für Metformin entwickelt, welches die Quantifizierung von Metformin in Mausgewebe und Plasma ermöglicht. Die erhaltenen pharmakokinetischen Daten zeigen, dass der Einsatz des SGLT2 – Inhibitors die Pharmakokinetik von Metformin in Geweben und Plasma beeinflusst.

Abbreviations

AA	Amino acids
AC	Acylcarnitine
Ala	Alanine
alphaAAA	α-Aminoadipic acid
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BCAA	Branched chain amino acids
Cit	Citrulline
CN	Carnosinase
CoA	Coenzyme A
CV	Coefficients of variation
CYP	Cytochrome P450
Cys	Cysteine
DNA	Deoxyribonucleic acid
eNOS	Endothelia nitric oxide synthase
FIA	Flow injection analysis

GC	Gas chromatography
Gln	Glutamine
Glu	Glutamic acid
GLUT	Glucose transporter
GLUT2	Glucose transporter 2
Gly	Glycine
HbA1c	Glycated hemoglobin A1c
HDL	High-density lipoprotein
His	Histidine
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
Ile	Isoleucine
LC	Liquid chromatography
LDL	Low-density lipoprotein
Leu	Leucine
LOD	Limit of detection
LOQ	Limit of quantification
LPLAT	Lysophospholipid acyltransferase
Lys	Lysine
lysoPC	Lysophosphatidylcholine
Met	Methionine
MetSO	Methionine sulfoxide
MRM	Multiple reaction monitoring
MS	Mass spectrometry

MS/MS	Tandem mass spectrometry
OCT	Organic cation transporter
Orn	Ornithine
PC	Phosphatidylcholine
Phe	Phenylalanine
PLA2	Phospholipase A2
PPAR-α	Peroxisome proliferator-activated receptor-α
Pro	Proline
PUFA	Poly unsaturated fatty acids
RT	Room temperature
SGLT	Sodium/glucose cotransporter
SGLT1	Sodium/glucose cotransporter 1
SGLT2	Sodium/glucose cotransporter 2
SGLT2I	SGLT2-inhibitor
SM	Sphingomyelin
T2D	Type 2 diabetes
Thr	Threonine
TNF	Tumor necrosis factor
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

1. Introduction

1.1. Metabolic syndrome and diabetes

The metabolic syndrome is a composition of dysfunctions which increases the chance of developing diabetes type 2 (T2D) or cardiovascular diseases [1]. It is defined as the accumulation of multiple factors, in detail, insulin resistance, impaired glucose tolerance, hyperinsulinemia, dyslipidemia, and hypertension [2] and abdominal obesity [1]. However, insulin resistance might be the major driving factor resulting in the further dysfunctions [3]. But the actual cause of the metabolic syndrome and diabetes is still under discussion [4]. Since its first description the metabolic syndrome undergoes a variety of changes in its definition. Currently the used definition comprises the first proposed factors and in addition abdominal obesity measured by waist circumference [1].

Diabetes mellitus is the collective term for a group of chronic metabolic disorders, predominantly characterized by an impaired glucose tolerance. The enduring high glucose levels are associated with dysfunction of eyes, kidneys and the cardiovascular system. The two major types of diabetes with the highest incidence rates are type 1 diabetes, and type 2 diabetes. In case of type 1 diabetes the pancreatic ß-cells fail to produce a sufficient amount of insulin, thereby causing an impaired glucose response. In contrast T2D is characterized by insulin resistance, which results in a lack of response to elevated insulin levels and consequently to impaired glucose tolerance [5]. By affecting about 380 million adults worldwide, T2D has become a challenge to public healthcare systems [6] and the estimated incidence rate as well as the economic costs are still rising [7]. In this context, Germany is no exception. In 2014 it was assumed that approximately 4.6 million adults have diabetes [8]. The number of undiagnosed or pre-diabetic people is estimated to be approximately 50% [9]–[11]. This increase in diabetes is forced by a combination of massive changes in lifestyle, increased food intake and lesser physical activity, as well as population aging. Therefore, new T2D therapies targeting a wide range of targets, have been identified and are under investigation [12], [13]. Despite these efforts, single drug treatment does not seem to be the optimal way to treat a multifactor disease like T2D.

The primary management of diabetes patients includes diet and exercise, followed by first line pharmacological treatment with metformin. The basic aim of the treatment is to achieve long-term control of blood glucose levels. If this approach to control blood glucose levels fails, metformin should be complemented by a second pharmacological agent [14]. In this regard, metformin and inhibitors of renal SGLT2 transporters have been proven to be an effective combination.

Pharmacological interventions focus on the control of blood glucose levels. However, functional dysregulations of metabolic processes, assoziated with diabetes, are not part of the clinical diabetes mangement. As these dysregulations are correleted with insuline sensitivity, arterosclerosis, heart disease, hypertension and diabetes[15] the affected metabolic pathways which leads to the primary and secondary pathological characteristics are not completely understood.

1.2. Treatment of type 2 diabetes

1.2.1. Metformin: Molecular mode of action

1,1-dimethylbiguanide, commonly known as metformin, belongs to the substance class of biguanides. Its antidiabetic action is known since 1929 [16] and since it has become the first choice in modern T2D care [17]. Furthermore, metformin has been suggested as potential treatment for polycystic ovary syndrome (PCOS) [18], cancer [19], [20] and gestational diabetes [21], [22]. It is also reported to reduce the severity of fatty liver disease, and to lower the risk of vascular complications associated with T2D [23].

Its primary mode of action is a decrease of gluconeogenetic activity in the liver [24], [25]. In addition to its primary action as glucose lowering agent, metformin modulates a broad range of metabolic pathways. It was shown to act as insulin sensitizer in liver and peripheral tissues [26], [27], which might be due to its impact on insulin receptor expression [28].

One of the molecular actions of metformin is a mild inhibition of the mitochondrial complex I [29] causing an increase in the AMP/ATP ratio. AMP binding to AMPK is one of the prerequisites for AMPK activation [30], [31]. AMPK regulates the cellular energy status and is involved in maintaining cellular functions under energy-restricted conditions. Metformin induced activation of hepatic AMPK results in increased fatty acid oxidation and inhibition of lipogenesis, gluconeogenesis, as well as inhibition of cholesterol and protein synthesis in the liver. Generally, switching the cells from anabolic to catabolic status, AMPK limits the consumption of ATP and increases ATP production [32], leading to an elevation in β-oxidation and glucose uptake.
Next to liver, which is a major player in antidiabetic drug action, similar effects of the complex I inhibition were found to be present in skeletal muscle [33], pancreatic β-cells [34], neurons [35], and lung [36] probably indicating a metformin induced regulation of the energy status in peripheral organs via an AMPK dependent pathway. However, metformin was also shown to cause an AMPK-independent up-regulation of the Akt survival pathway [37]. As reported by Maida *et al.* [38], metformin treatment correlates with increased plasma levels of glucagon-like peptide-1 (GLP-1) and

modulates multiple components of the incretin axis via a peroxisome proliferator-activated receptor-α (PPAR-α) depended mechanism.

Although, massive efforts to elucidate molecular metformin action have been made, its mechanisms are not yet fully understood. In this context, most studies on diabetes and metformin action focus on the central metabolic organs, liver, skeletal muscle, adipose tissue, kidney and the intestinal tract and thereby disregard the fact, that the impact of diabetes and metformin is not restricted to the main metabolic sites of the body, like the examples from retinopathy and the breast cancer risk has shown. Therefore, the elucidation of diabetes induced metabolic action as well as the effect of pharmacological interventions in endocrine organs (adrenal gland), reproductive tissues (testis) central, respiratory sites (lung), and the nervous system (cerebellum) is of vital importance.

1.2.2. Sodium glucose co-transporter 2 inhibitor

As T2D is a progressive disease which involves multiple metabolic defects, metformin monotherapy may not be sufficient to maintain glycaemic control over time. The use of a combination of antidiabetic drugs with different modes of action can overcome compensatory effects in T2D monotherapy. Additionally, it has the potential to have synergistic effects and therefore enhances glycemic control and reduces the development of T2D related diseases [39].

One of the most promising target for a combination therapy is the sodium/glucose co-transporter (SGLT) family, as it is the major transporter family for glucose reabsorbtion in the proximal tubulus of the kidney. Different subtypes of sodium glucose co-transporters (SGLT 1-6) exist, of which only SGLT1, SGLT2 are described as active glucose transporters [40]. In contrast, the passive cellular glucose uptake is facilitated by transporters of the GLUT family. SGLT2 is expressed mainly in kidney, and in lower extent in brain, liver, thyroid, muscle and heart [40]. Amongst other site of action SGLT1 and SGLT2 facilitate glucose reabsorption into the cells of the proximal tubule of the kidney. Together they are reabsorbing 99% of the excreted glucose. In the healthy kidney SGLT2 accounts for 90% of the reabsortion and SGLT1 only for 10% [41]. Together with the GLUT2 transporter SGLT facilitate the glucose transport from the urine over the tubular cells to the blood [42]. As SGLT transporters are the first line actors in glucose reabsorbtion they became a target of therapeutic interest in treatment of diabetes.

Inhibition of the SGLT2 transporter in the kidney leads to massive glucosuria and therefore to an insulin independent reduction in blood glucose levels. In mouse models and clinical trials the currently investigated SGLT2-inhibitors only diminish levels of glucose reabsorption by 50%. The unimpaired

action of the SGLT1-transporter is responsible for the observed residual glucose absorption which is may enhanced when SGLT2 transport is inhibited [43], [44].

No pharmacokinetic interferences of SGLT2-inhibitors with other wildly used antidiabetic drugs have been described [45], rendering this drug class a reasonable choice for second-line therapy [39], especially in combination with metformin [39].
Several SGLT2-inhibitors are already available in some countries (canagliflozin, dapagliflozin, empagliflozin, ipragliflozin, and tofogliflozin) and some others are candidates for clinical applications (ertugliflozin, phlorizin, remogliflozin, and sergliflozin). Their main mode of action is to compete with D-glucose for binding to the SGLT transporter in the kidney and therefore avoid the reabsorbtion of glucose [46].

Phlorizin is a long known inhibitor of SGLTs and played an important role in the development of newer, more selective SGLT-inhibitors. It was shown to efficiently inhibit SGLT2, but also affects SGLT1 related glucose transport and is associated with serious side effects like hypoglycemia. In contrast, the SGLT2 inhibitor AVE2268, a substituted glycopyranosid, selectively inhibits the SGLT2 transporter causing decreased glucose reabsorbtion together with a lower risk of hypoglycaemia [47].

SGLT2-inhibitors mimic carbohydrate restriction by the acute decrease of blood glucose levels, leading to a change in global energy status of the body and thereby has an immediate impact on the metabolism. SGLT2-inhibitors causing a reduced glucotoxicity in the liver in combination with an enhanced influx of glucose into the liver [48]. In 2008 [48] published that endogenous glucose production was reduced in Dapglifolzin treated ZDF rats. In contradiction [49], [50] observed an increase of endogenous glucose production in men. They also showed that the SGLT2-inhibitor treatment improves insulin sensitivity and β-cell function, and a shift from carbohydrate to lipid utilization together white a rise in lipid oxidation.

1.3. Metabolomics

Diabetes and the metabolic syndrome are disorders of the metabolism. The characterization of the occurring metabolic changes is the key for understanding the molecular mechanisms behind them. Metabolomics is one of the tools to answer this question and has already shown its power in diabetes research in several studies [51], [52].

Metabolomics aims to identify and quantify the total assemblage of small molecules (<1500 Da) in a biological sample. Therefore, metabolomics tries to cover the sum of all biochemical processes in living organisms. Even if the high-throughput analysis of hundreds of metabolites is a relative new

field in biological and clinical sciences, the knowledge for the correlation of metabolites and human diseases has taken place for centuries. Two mile stones in unfolding the metabolic network were the elucidation of the urea cycle 1932 and the tricarboxylic acid cycle 1937 and the continuous work on both of these central metabolic cycles [53], [54]. Modern metabolomics technology is based on the development of the first mass spectrometer at the beginning of the 20th century by J.J. Thomson [55], [56]. After a gap of 60 years crucial advancements in mass spectrometry technology offer new possibilities for scientist and mass spectrometry based metabolite measurements begin to attract an ever increasing attention across the scientific community [57]–[60]. In 1998 Stephen Oliver coined the term metabolomics and showed the enormous potential of joining metabolomics to genomics and proteomic approaches [61]. Like the other “omics”, data obtained with metabolomics technologies can be used to deduce regulatory networks from a cellular basis up to the biological system level. In this way, metabolomics is the consequential progression from investigating gene expression to the analysis of finally appearing phenotypes and can provide evidence for changes in biochemical pathway.

While in genomics and proteomics databases for sequence information are readily available since over two decades, such a concept has only been introduced for metabolomics. With the human metabolome database (HMDB) [62]–[64] information about structural and physicochemical properties, biofluid concentrations, association with disease and pathway information are available for many metabolites. Additionally, the LIPID MAPS Lipidomics Gateway database provide a growing number of information and tools for lipid research [65]–[67]. However, the metabolome of an organism is constantly changing. The biochemical variance occurs due to the metabolic response, influenced by circadian and seasonal rhythms, genotype, sex, age, drugs, and environmental impact leading to differences in the metabolomic phenotypes. For this reason, no absolute statement about metabolite concentrations can be made.

However, ranges of metabolites concentration for healthy and diseases status can be defined, and ratios between closely related metabolites give insight into biochemical pathways opening the path for the detection of biomarkers.

This work is based on a targeted metabolomics approach. Targeted metabolomics deals with specific metabolite classes or specific pathways, and allows their absolute and precise quantification. The progressions in method development, which took place within the last couple of years, led to the optimization of the performance of metabolomics analyses, in regard to crucial parameters. However, there still remain a couple of major issues unique to metabolomics approaches (not given with other “omic” approches). The HMDB [64] lists over 40,000 putative metabolites, which do not generally share the same chemical or physical properties and in addition exhibit quite different levels of abundance depending on analyte, organism or matrix. No single method can extract, separate, ionize and detect all of them. Therefore, a broad range of different MS application setups were developed to optimize the extraction, detection and quantification of a subset of analytes of interest.

Mass spectrometry based applications can be grouped into two major approaches 1.) Flow injection analysis (FIA) uses direct infusion of the sample and 2.) MS-analysis coupled to an a priori separation technique, either gas chromatography (GC) or liquid chromatography (LC). Both methods have their advantages and limitations. The flow injection analysis (FIA) introduces complex biological samples directly into the mass spectrometer, allowing for fast, cheap, and high-throughput suitable sample analysis. FIA-MS provides a metabolite fingerprinting tool for application in targeted and untargeted metabolomics and is used in a wide range of applications [68]. However isobaric metabolites (metabolites with the same nominal mass) cannot be separated in a FIA based approach.

Coupling a separation step (like liquid chromatography) to a MS analysis reduces the complexity of the samples. Thus, the reduction in matrix indifferences, due to the reduction in noise level and improved ionization, leads to enhanced selectivity and sensitivity. Furthermore, analytes with the same nominal mass but different physical properties can be separated previous to analysis, enabling the discrimination of isobaric metabolites. However, with typical run times of 10 to 60 min the HPLC based separation step might be a bottle neck in the analysis and limited sample throughput. But development continues and new Ultra High Performance Liquid Chromatography systems shorten the analysis time making it more feasible for high-throughput analysis.

1.4. Metabolite extraction

The choice of the sample preparation method is extremely important for metabolomics studies. The chemical and physical diversity of the metabolites and the fast dynamics of their turnover make metabolite extraction a challenge. The first step in sample preparation is the immediate quenching of the metabolism, to stop metabolite turnover and degradation [69]. Quenching conditions, like low temperature and pH should be conserved during the extraction process.

The second critical step is the extraction of metabolites from the biological sample. The extraction solvent used during sample preparation not only influences the ionization behavior of the metabolites but highly contributes to the number of extracted metabolites and the range of extracted metabolite classes. An optimal extraction solvent should extract as many metabolites as possible with equal efficiency while avoiding chemical or physical degradation and therefore ensuring an optimal representation of the metabolic fingerprint of an analyzed system [69]. Facing the high chemical and physical diversity of metabolites the extraction solvent of choice should solubilize both, polar and nonpolar analytes. Furthermore, it should minimize matrix effects and maximize the reproducibility of the quantification.

1.5. Metformin quantification in metabolomics samples

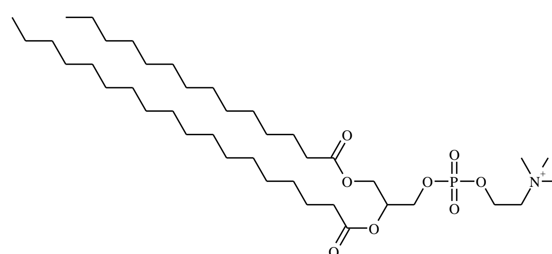
Metformin is handled as the gold standard in T2D treatment [70]. Therefore absolute quantification of metformin is crucial in basic research as well as in clinical sciences. Radioisotope labeled [71], HPLC [72], [73], and LC-MS [74]–[77] approaches have been undertaken. Although based on different methods their major focus remained the same: metformin quantification in tissues [71], [72] and biofluids [73]–[77]. However, for metabolomics special sample handling and preprocessing of tissues is required [78], [79]. Therefore, my goal was to develop a LC-MS/MS quantification method for the analysis of metformin from samples preprocessed for metabolomics analysis.

1.6. Metabolite classes

The applied targeted analysis with the Absolute*IDQ*™ kit p180 allows for the identification of several metabolite classes, which belong to different biochemical pathways and present a wide range of chemical and physical properties. For a couple of metabolites within the investigated panel (e.g. hexoses), a correlation between their levels and the metabolic syndrome as well as diabetes and its treatment has already been shown. However, a wide range of metabolites, their potential correlations and feasibility as biomarkers have not yet been addressed.

1.6.1. Phosphatidylcholines

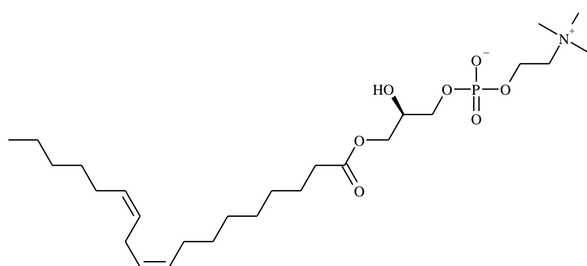
Phosphatidylcholines (PCs) are the major building block of cellular membranes, and main component of plasma lipoproteins, bile and lung surfactant. Furthermore, they are precursors of different cellular signaling molecules [80]. It has long been known that changes in PC composition are associated with diabetes [81]. Some recent publications show the correlation between diabetes, cardiovascular disease and several specific PCs [82], [83].



Phosphatidylcholines belong to the class of phospholipids. They consist of a choline head group coupled to a glycerol backbone. Generally, a saturated fatty acid is bound to the first carbon of the

glycerol, an unsaturated fatty acid to the second carbon and a phosphate group to the third. The fatty acids are variable in their chain length as well as in their saturation status. The applied targeted metabolomics approach allows for the determination of the sum of carbon atoms and double bounds of both fatty acid side chains and the identification of the bond type with which those side chains are linked to the phosphatidylcholine head group (diacyl (aa), acyl-alkyl (ae) or alkanyl).

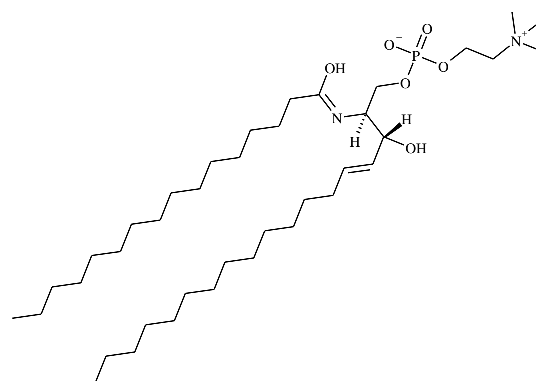
1.6.2. Lysophosphatidylcholines



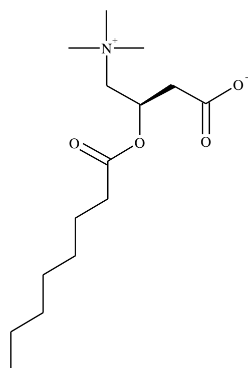
Lysophosphatidylcholines (lysoPCs) are biochemical closely related to phosphatidylcholines. They are the product of partial hydrolysed PCs by the enzymatic action of phosphorlipase A2 (PLA2). PLA2 removes the fatty acid from the second carbon atom of the PC glycerol backbone, resulting in a lysoPC and a free fatty acid. As PCs and the action of PLA2 are correlated with diabetes and cardiovascular disease, consequently lysoPCs levels are also recognized to be changed in these diseases [84], [85].

1.6.3. Sphingomyelin

Sphingomyelins (SM) belong to the class of sphingolipids. They are a crucial part of all cell membranes, especially in the myelin sheath of neurons, but they appear also in lipoproteins and other membrane like structures. Chemical they consist of a phosphate group linked to a sphingosin backbone together with an acyl linked variable fatty acid. Whether or not the fatty acid chain length or saturation status dedicate a biological function has not yet been investigated [86]. However, SM containing saturated fatty acids acyl chains alter membrane integrity and function [87] and are correlated with insulin resistance, obesity and metabolic syndrome [88].

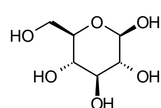


1.6.4. Acylcarnitines



Fatty acids are essential for the building of more complex lipids, as energy fuel for the cell, and as nuclear signaling molecules. As lipophilic molecules they generally actively transported though the cellular membranes [89]. The transport of fatty acids across membranes (especially the mitochondrial membrane) is facilitated by transporters which transport fatty acids bound to carnitine. These acylcarnitines (AC) are generated from coenzyme A (CoA) activated fatty acids which are bound to carnitine by an exchange of CoA for carnitine via carnitine palmitoyltransferase I. The formation of fatty acid CoA esters and their transport through the mitochondrial membrane as acylcarnitines is crucial for a proper beta-oxidation. The bulk of CoAs and therefore also of acylcarnitines derived from fatty acid oxidation, but they can be formed from almost any CoA ester, such as ketone bodies, forming C4-OH-carnitine [90], or the degradation products of tryptophan, lysine (hydroxybutyrylcarnitine, glutarylcarnitine) [91], and the branched-chain amino acids (valine, leucine, isoleucine), which results in the formation of C3- and C5-carnitines (propionyl- methylmalonyl-, isovaleryl-carnitine) [92].

1.6.5. Hexoses



Diabetes mellitus is primarily described by an abnormal glucose metabolism and the enhanced excretion of glucose via urine. Therefore, blood glucoses levels, and for long-term monitoring glycated hemoglobin levels, are used as clinical readout parameter. Other hexoses, like galactose, mannose, and fructose are related to insulin signaling [93] and in recent years were set into the focus of diabetes research, among other things, for their role in protein glycosylation [94] and glycemic control [17].

Cellular glucose transport and utilization are closely related to fatty acid levels, because both are cellular energy sources. In the states of insulin resistance and T2D, increased levels of free fatty acids inhibit glucose uptake and utilization and therefore in part responsible for this pathophysiological state [95]. The Absolute*IDQ*™ p180 kit method is not resolving the different hexose species (e.g. glucose, fructose, galactose, and mannose). Therefore, only the sum of all appearing hexoses in a sample is measured with the p180 kit.

1.6.6. Amino acids and biogenic amines

The proteinogenic amino acids and other biogenic amines, even if similar in chemical structure are involved in a quite different range of biochemical pathways. They are building blocks for proteins and precursors for a wide range of basic cellular biochemical reactions. These ranges from the synthesis of purines, pyrimidines, nukleotids and urea, to the formation of citric acid cycle metabolite. They are involved in the methylation of DNA and variety of other compounds [96]. The so called glucogenic amino acids can be converted into glucose for gluconeogenesis. Alanine is the main glucogenic amino acid, released from muscle during fasting or energy shortage, and used for gluconeogenesis in the liver [97]. Finally branched chained amino acids (BCAA) are linked to the synthesis of fatty acids and by this to insulin resistance and diabetes [98]. In obese and diabetic subjects, plasma BCAA levels are increased and correlate directly with insulin levels [99], [100]. Additionally, BCAAs are one source for the alanine production in muscle and therefore contribute to gluconeogenesis [97].

1.6.7. Nomenclature of lipids

The following nomenclature for metabolites is used in this thesis.

For phosphatidylcholines: PC *bound type* (*sum of carbon atoms of the side chains*:*sum of double bonds of the side chains*). For example, PCaa (32:2) denominates a diacyl PC with a sum of 32 carbons in both side chains and two double bounds.
For lysophosphatidylcholines: lysoPC (*sum of carbon atoms in the side chain*:*sum of double bonds of the side chain*).
For sphingomyelin: SM (*sum of carbon atoms in the side chain*:*sum of double bonds of the side chain).*
For acylcarnitines: C (*sum of carbon atoms of the fatty acid chain*:*sum of double bonds of the fatty acid chain).* Additionally, the fatty acids can contain dicarboxy (DC), methyl (M), or hydroxyl (OH) groups. Further, the applied metabolomics approaches do not allow the separation of isobaric metabolite pairs. These pairs are denominated e.g. as C4-OH(C3DC), meaning these are either hydroxybutyrylcarnitine (C4-OH) or malonylcarnitine (C3DC).

1.7. Aims of this thesis

The overall object of this thesis was to extent the knowledge of systemic and organ specific pharmacokinetic of metformin and the mode of action of drug interventions in the complex disease diabetes by studying the affected biochemical pathways. To reach this aims targeted mass spectrometry approaches should be applied. The metabolic patterns of drug intervention should be analyzed in different organs and plasma from a diabetic mouse model treated with antidiabetic agents (metformin and a SGLT2-inhibitor) to identify biochemical pathway affected by different treatment strategies. The effect of the drug intervention on the metabolism should be examined by statistical analysis of the results to find characteristic patterns of antidiabetic treatment or identify single metabolites or biochemical pathways affected by the intervention. Therefore, not only single metabolite changes should be investigated but also metabolite ratios should be taken into account to identify biochemical alterations.

2. Material and Methods

2.1. Chemical and reagents

Chemical	Manufacturer
Acetonitrile	Roth
Ammonium Acetate	Sigma-Aldrich
AVE2268	Sanofi-Aventis
Ethanol	Merck
Formic Acid	Fluka
Hydroxyethylcellulose	BASF
Metformin	Sigma-Aldrich
Metformin-d6	Toronto Research Chemicals
Methanol (HPLC grade)	AppliChem
MilliQ H2O	Millipore
Phenylisothiocyanate	Roth
Phosphate buffered saline	Sigma Aldrich
Pyridine	Roth

2.2. Animal study

All animal protocols and procedures were approved by the Government of Upper Bavaria (reference no. 55.2-1-54-2531-70-07). Mice were bread and maintained in the German Mouse Clinic, Helmholtz Zentrum München in accordance to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm). The study was based on db/db mice (db/db [BKS.Cg-m+/+Leprdb/db] and wild-type mice [BKS.Cg-m+/+Leprm/m]; Jackson Laboratories, Bar Harbor, ME, USA) fed with a high-fat diet postweaning (S0372-E010, ssniff Spezialdiäten). The mice were housed in single ventilated cages (SealsafePlus AERO IVC Greenline, Tecniplast) at 22±1 °C and controlled humidity with a 12/12 h light/dark cycle.

The db/db mice harbour an abnormal splice junction variant preventing the transcription of a leptin receptor “long” isoform (Leprdb-1J). When bred on the C57BLKS/J (BKS) genetic background, obese

db/db mice develop early onset diabetes paralleled by pancreatic β-cell atrophy. A High Fat/High Carb diet from the age of 3 weeks induced a markedly diabetic phenotype in all mice at experimental start (8 weeks of age ± 3 days). All diabetic groups were body weight and blood glucose matched (body weight 47.3 ± 2.1 g; blood glucose 27.0 ± 4.2 mmol/l).

2.2.1. Animal treatment and treatment groups

Adult, male mice (8 week old) were randomly assigned to 10 treatment groups (Table 1). Five groups with daily drug treatment over 14 days (sub-chronic) and five groups with a single drug dose for 4 hours. The db/db mice were treated either with metformin (300 mg kg^{-1} day^{-1}; Sigma Aldrich), SGLT2-Inhibitor (30 mg kg^{-1} day^{-1}; AVE2268, Sanofi Aventis), a combination of both (with the same concentrations as in the single treatment), or the drug vehicle solution (95% hydroxyethylcellulose 250G Pharm, Fagron; 5% Solutol HS15, BASF). Wildtype mice were treated with vehicle solution. The drugs were dissolved in 0.5% vehicle solution and applied by gavage. Study related animal maintenance and treatment was carried out by Markus Scheerer and Susanne Neschen.
To ensure the diabetic status, inclusion criteria for db/db mice at the beginning of the drug treatment were weight greater than 44 g and blood glucose (measured with Contour, Bayer Vital) above 19.43 mmol/l.

Table 1: Animal treatment groups
Acute treated mice received a single drug or vehicle dose, the sub-chronic group received a daily drug or vehicle dose over 14 days.

Mouse	Acute treatment 4 hours	n	Sub-chronic treatment 14 days	n
wt	Vehicle	10	Vehicle	10
db/db	Vehicle	10	Vehicle	10
db/db	Metformin	10	Metformin	10
db/db	SGLT2-Inhibitor	10	SGLT2-Inhibitor	10
db/db	Combination	10	Combination	10

2.2.2. Sample material collection from animals

Mice were fasted 4 hours and killed with an isoflurane overdose 4 hours after the single treatment or 18 hours after the last 14 day treatment. The skin of the mice was moistened with ethanol and the abdominal wall was opened. All available blood was drawn from the *vena cava* into 1.5 ml tube and immediately centrifuged (10.000 × g, 2 min, 4 °C). The plasma was transferred to a new vial frozen in liquid nitrogen and stored at −80 °C. The organs were dissected, frozen in liquid nitrogen and stored at −80 °C until further processing. Organ withdrawal was done in corporation with Markus Scheerer, Susanne Neschen, Alesia Walker, Barbara Pfitzner, and Gabriele Zieglmeier.

2.2.3. Animal sample preparation for metabolomics measurements

Tissue or Organ samples were weighted and transferred to pre-cooled homogenization vials (Precellys® Homogenization Kit, Peqlab) which contained ceramic beads (1.4 mm diameter). The appropriated pre-cooled extraction solvent was added in a distinct ratio as shown in Table 2. Tissue homogenization was performed with a Precellys® 24 homogenizer (Peqlab) with an integrated cooling unit. Each 20 s homogenization interval (5500 rpm, −4 °C) was interrupted by a 30 s pause interval (−4 °C). Finally, the samples were centrifuged (2300 × g, 5 min, 4 °C) and the supernatants were used for metabolomics measurements. Plasma samples were thawed at RT and centrifuged (2750 × g, 2 min, 4 °C). All homogenization and centrifugation steps taken place immediately before the metabolomics measurements.

Table 2: Extraction solvents of analyzed animal tissue types

Tissue	Extraction solvent	µl solvent/ mg tissue
Adrenal gland	ethanol/phosphate buffer (85/15; v/v)	12
Cerebellum	ethanol/phosphate buffer (85/15; v/v)	6
Fat (visceral)	ethanol/phosphate buffer (85/15; v/v)	6
Kidney	methanol (100%)	3
Lung	ethanol/phosphate buffer (85/15; v/v)	3
Liver	ethanol/phosphate buffer (85/15; v/v)	3
Testis	methanol (100%)	6

2.3. Metabolomics analysis

2.3.1. Absolute*IDQ*™ kit p180

The Absolute*IDQ*™ kit p180 from Biocrates (referred to as p180-kit) was performed according to the manufacturer's manual (UM-P180). The calibrators for the LC-MS/MS method were pipetted onto the filter inserts of the p180-kit 96-well filter plate which contains the internal standards for the FIA-MS/MS measurement, and dried under a continuous nitrogen stream (Ultravap nitrogen evaporator, Porvair Sciences, Leatherhead, U.K.) for 30 min. In the next step, 10 µl of each sample fluid (mouse tissue extract, mouse plasma, cell culture samples, cell culture supernatant, reference plasma, quality controls, calibrators) were transferred to the p180-kit 96-well filter plate. The samples were dried under a continuous nitrogen stream (30 min). Primary and secondary amines were derivatized (20 min) with 5% phenylisotiocyantate (Sigma Aldrich) in ethanol/water/pyridin (1/1/1; v/v/v) and dried under a nitrogen stream (45 min). Extraction of metabolites and internal standards were performed under continuous shaking for 30 min with methanol/amonium acetate (5 mM) and the 96-well filter plate was subsequently centrifuged (500 × g, 5 min). The obtained extraction solution was split into two parts. On part was diluted in H_2O (1/1; v/v) and further used for LC-MS/MS analysis, the other part was diluted in biocrates running solvent (1/5; v/v) to perform FIA-MS/MS analysis. All solutions, solvents and internal standards pipetting steps were performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland).

For amino acids and biogenic amines measured by LC-MS/MS, a HPLC separation was performed on a 1200 Series HPLC system (Agilent) on a XDBC18 column (3 × 100 mm, 3,5 µm) with a C18 pre-column (Agilent) running an acetonitrile/water gradient containing 0.2% formic acid.
Metabolites from the classes of acylcarnitines, phosphatidylcholines, lyso-phosphatidylcholines, sphingomyelins and the sum of hexoses were measured by flow injection (FIA-MS/MS) running with the p180-kit running solvent. Samples were injected by an PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland) to a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany). The analytes were ionized in a TurboV™ ESI source and detected by multiple reaction monitoring (MRM) using an API4000 mass spectrometer (Sciex Deutschland GmbH, Darmstadt, Germany). Autosampler and mass spectrometer were controlled by the Analyst software (1.5.1 or 1.6). Processing of the raw data for quantification and quality assessment were done with the Met*IDQ*™ software package.

2.4. Mass spectrometry based metformin quantification

FIA-MS/MS was performed by injecting 5 µl aliquots of each sample to am isocratic mobile phase. The composition of solvent A was 5/95 methanol/water (v/v), solvent B was 90/10/0.05 methanol/water/formic acid (v/v/v). The solvents were mixed online 90/10 and delivered, with a flow rate of 0.7 ml×min^{-1} into the mass spectrometer. To protect the MS against contaminations a C18 guard column (4 mm × 2mm) (Phenomenex) was used.

Optimization of ion source and MS parameters and determination of mass transitions for multiple reaction monitoring (MRM) were performed using the Analyst software compound optimization tool. The parameters were presented in Table 3.

The HPLC system consisted of one binary gradient HPLC pump (LC-20AB), an autosampler (SIL-20AC), and matching controlling unit (CBM-20A) (all from Shimadzu) and a 4000 QTrap triple quadrupole mass spectrometer with an Turbo V electro spray ionisation source (AB Sciex Deutschland GmbH, Germany). System control and analytical data analysis were processed by Analyst software 1.5.1.

Table 3: MRM parameter of metformin and deuterated metformin
Ion source parameters for electrospray ionization in positive ionization mode were optimized for two Q3 masses for metformin (#1,#2), as well as for deuterated metformin (d6#1, d6#2) with the following results. The ion spray voltage was set at 5500 V. The common parameters nebulizer gas (gas1) and auxiliary gas (gas2) were set to 80 and 15 p.s.i., respectively. The curtain gas was set to 15 p.s.i., CAD was set on medium, inter face heater was set to ON, source temperature (TEM) was set to 500 °C. DP = declustering potential, EP = entrance potential, CE = collision energy, CYP = cell exit potential. Dwell time for all compounds were set to 100 ms.

	Metformin #1	Metformin #2	Metformin d6 #1	Metformin d6 #2
Q1 Maas (Da)	130.14	130.14	136.13	136.13
Q3 Maas (Da)	60	71.1	60.2	94.1
DP	35	35	50	48
EP	8	12	8	8
CE	18	34	22	22
CXP	4	2	4	4

2.4.1. Metformin quantification in mouse samples

For quantification of metformin concentration in tissues, the same samples as for the metabolomics analysis were used. For sample preparation see chapter 2.2.3. 2 µl auf each sample was diluted 1:10000 in methanol containing metformin-d6 as internal standard. The final concentration of metformin-d6 was 5 ng/ml. As quality control 3 ng/ml metformin in methanol with internal standard were processed in parallel to the samples.
For the quantification of metformin in plasma, 5 µl plasma were diluted 1:500 in methanol containing a final concentration of 5 ng/ml metformin-d6 as internal standard.

2.5. Statistics

The R software environment for statistical analysis (Version 3.0.2) [101] was used for data processing and statistical analysis.

Determination of statistical differences was done for single metabolite concentrations and all possible ratios of them. Additionally concentration and ratios of whole metabolite classes and sub-classes were tested for differences.

For statistical evaluation a [0,1] normalized linear model was applied to each pair of treatment or phenotype group. The coefficient of determination (r^2), p-values, q-values, p-gains and the slop (β) were calculated on the linear model, using log-scaled data. Additionally, the confidence intervals of the difference of means for all metabolite concentrations and concentration ratios were calculated on not log-transformed data. As measure of the effect size, the Glass's Δ were calculated on not log-transformed data.

Development of the statistical methods was done in corporation with Jörn Leonhardt and Gabi Kastenmüller from the Institute of Bioinformatics and Systems Biology.

2.5.1. p-values or confidence intervals

The use of p-values is a widely accepted method for the justification of the "relevance" of a scientific experiment or a clinical study. Despite this acceptance in the scientific community there are some pitfalls in the use of p-values for study justification. A disadvantage of the p-value is the fixation on the indication of the significance of study results; ignoring the biological or clinical relevance. Beside this, statistical tests are very sensitive to the number of observations. A small number of observations probably do not identify even large effects. Whereas, large sample numbers can provide significant results on very small or worthless effects [102]. Another disadvantage is the limitation of meaningfulness of p-values for small sample groups. The p-value should be a reliable measure for the strength of evidence, meaning repeating the experiment leading to same p-values. But p-values arise by chance because they are depending on the effect size and the standard error of the observed samples and both parameters itself are variables if once investigate multiple samples from the same distribution. This leads to different p-values for a repeated experiment, even when small sample numbers were tested.
In contrast, confidence intervals are less variable then p-values and they indicate the magnitude of possible variations which might occur in replicated experiments.
An overview to the rich discussion in the scientific community is given in the introduction of [103], which shows the potential of combining significance test and confidence intervals. Presenting both, confidence intervals and p-values provides more arguments for the justification of experimental results than p-values alone.

2.5.2. Distribution assumption

Statistical methods often make assumptions on the distribution of the data. For this reason samples should be tested for their distribution prior to statistical tests. But tests on normal distribution have insufficient power to discriminate between different distributions on small sample sizes (n=10) and so may not provide reliable information about the underlying distribution. However, most statistical methods which work on normal distributed data also fit well to quasi-normal distributions. For the here investigated metabolites, [104] show that the log distribution fit best to the distribution of most metabolite ratios in a human cohort study, and they applied successfully linear regression models to log transformed metabolite ratio data. For this reason linear regression calculation in this work are also based on log-transformed metabolite concentration ratios.

[105] show that the calculation of confidence intervals from the geometric mean with log transformed data have smaller intervals compared to non-transformed data on the arithmetic mean. This is because the arithmetic mean is more prone to extreme values. Calculating the difference of means on a log scale, make a comparison of the results much more complex. Comparing means is simpler on non-transformed data [106]. For this reason confidence intervals were calculated on the arithmetic mean of non-transformed data.

For sample sizes smaller than 30 the assumption of a normal distribution of the data may not correct. A better estimator for the standard deviation of small sample sizes is the *t*-distribution. Therefore, confidence intervals are calculated assuming a t-distribution to find the critical values (number of standard errors to add and subtract from the margin of error (e.g. 95% CI)).

2.5.3. Metabolite ratios and p-gain

Ratios between metabolites may provide more information than the metabolite alone. A metabolite ratio can be a direct marker for an underlying biochemical process. For instance, C8/C6 and C8/C10 ratios found to be elevated in medium-chain acyl-CoA dehydrogenase deficiency patients [107], and different amino acid ratios are connected with metabolic disorders in newborn [108]. Additionally, multiple examples of associations with hypothesis free testing of phosphatidylcholines ratios were published [109]–[111].

To investigate if a ratio of metabolites provides more information than the single metabolites alone; the p-gain was introduced as an estimator of this information gain.

Correlation studies in plants reported high p-values indicate metabolite pairs which might be linked by metabolic pathways [112]. Altmaier et al [113] showed that gain in partial η^2 of ANOVA from metabolite ratios results in an information gain and in a reduction of variability. Based on this results [114] uses a similar approach, calculating the p-gain from the ratio of metabolite pairs. All three publications understand a gain in p-value as an indicator for a biochemical link between the metabolite pairs which are may be influenced by an external parameter (treatment, phenotype, pathological state). In this work the p-gain for every metabolite ratio is defined as:

$$p\text{-}gain = \frac{min(p_A|p_B)}{p_{\frac{A}{B}}}$$

where p_A denotes the p-value from metabolite A between two groups, p_B the p-value for metabolite B, respectively. $p_{A/B}$ denotes the p-value of the ratios between two groups. The p-gain allows the quantification of the decrease in p-values with the ratios compared to the single metabolites. A higher

p-gain for a metabolite ratio indicates that a low p-value for the ratio is due to a change in the ratio rather than a change in a single metabolite.

2.5.4. Effect size

Effect size is a measure of the magnitude or strength of an observation (e.g. response to a treatment). The effect size provides a scale free measurement of differences among observations [115]. Glass's Δ is reported here as the measure of effect size [116].

$$\text{Glass's } \Delta = \frac{\overline{x_1} - \overline{x_2}}{s_2}$$

where $\overline{x_1}$ and $\overline{x_2}$ were the means of the sample groups, and s_2 were the standard deviation of the control group.

The Glass's Δ assumes that all groups are compared to a control group and only the standard deviation of the control group is taken into account. The advantage of this approach is that effect size would be the same for equal means, allowing an easy comparison between groups.

2.5.5. Correction for multiple testing

For statistical methods testing against a null hypothesis or on a confidence interval there is a probability of having false positive results. These errors became more likely to occur with increasing test number. Correction is needed whenever results from multiple statistical tests have to be combined in a single conclusion. Controlling the type I error rates (false positive statistical inference) is mandatory to protect against wrong positive conclusion or minimize this risk to a defined level. However, one must not forget that lowering the cutoff for the p-value can also increase the number of false negatives [117].

Here two different approaches were applied to control false positive error rates deriving from multiple testing, q-values for linear models and Bonferroni correction for confidence intervals. For the correction of the results from linear regression models the positive false discovery rate was computed after Storey and Tibshirani [118] and the p-value and corresponding q-values (which incorporates correction for multiple testing) were calculated.

Multiple calculations of confidence intervals lead to a similar multiple testing problem as for other statistical tests. In contrast to the false discovery rate for p-values; here the false coverage rate of the

interval is of interest. Confidence intervals are typical calculated for a margin of error, usually for a 95% confidence. This margin of error was corrected after the conservative Bonferroni method so that each confidence interval was constructed at a $1-\alpha/m$ level, where α is the significance level and m is the number of observations.

As the p-gain is introduced only as estimator for an information gain between single metabolite and their corresponding ratios, there is so far no strict statistical criterion for the correction of p-gains. Petersen et al. [119] addressed this problem and showed that $B/(2*\alpha)$ is a conservative critical value for the p-gain, where B is the number of observations and α the significance level.
This assumption is made for lager sample size ($n \geq 100$). Petersen et al. showed the strong dependency of the p-gain from the sample size. For instance, they calculated the p-gain from 100, 500, or 1000 samples results in p-gains of 10^2, 10^5, and 10^{10}, respectively.
In this study 16100 metabolite ratios (for every group wise comparison) where compared, leading to a p-gain correction value of $16100/(2*0.05) = 16.1*10^4$. Based on the data in Petersen et al. the expected p-gain for this work with n=10 sample should be in the range 10^{-1} to 10^2. Applying the $B/(2*\alpha)$ rule will result in no meaningful p-gain values for the experiment of this work.
Correlating the q-values, effect sizes and slops auf the here investigated metabolite ratios with the p-gain, most of the results with meaningful changes show p-gains above 100, which is also the upper limit of the expected p-gain range. An analysis of the distribution of p-gains within the datasets showed that around 90% of all p-gains were below 100. In this work this value is not taken as strict criteria for correction or exclusions of data, but more as a guideline for justification of the results. Taking a so much different p-gain criteria in contrast to Petersen et al. make sense if the assumed much smaller biological variance of the litter mate mice under controlled conditions in contrast to a human study, and the different sample size were taken into account.

Comparing the q-values (<0.05) with the CIs, some of the CIs indicating a non-significant difference. This effect was caused due to the application of different correction methods for multiple testing. For statistical justification of significance, only the q-values (Appendix 6.1.1 and 6.1.2) were taken into account.

2.5.6. Outliers

An outlier is an observation that is distinctly different from other observations in the sample. Outliers may result from erroneous sample handling or measuring, but they also occur due to random variations in biological processes or from the technical variance within the measurement process, or a combination of this.

Outliers are often defined as data points which are more than 3 times of the standard deviation σ away from the sample mean [120]. However, the mean and the standard-deviation are itself sensitive to outliers as the mean moves towards the outlier and the standard deviation is inflated. Therefore, in this thesis Hample’s outlier detection method was used. It replaces the mean with the median and the standard deviation with the median absolute deviation, which are both are more outlier-resistant alternatives [121], [122].
Detected outliers were removed and not replaced by sample mean or by possible data, since both options may alters the likelihood of a type-I-error in small datasets [123].

2.5.7. Statistic quality control

Metabolites where be measured in different quality and quantity within the individual experimental groups. Not all metabolites were measureable in all groups or all samples of a group. Some metabolites were just near the limit of detection and cannot be quantified in all samples of a group or not showed up in all samples between the treatment groups or phenotypes. Statistical analysis and data evaluation were performed only between groups with at least 3 valid samples in every group, following the standard for chemistry analysis in metabolomics studies [124].

2.5.8. Data pre-processing

As described in paragraph 2.2.3, each tissue was homogenized in a defined amount of solvent per milligram tissue. Therefore, a normalization of metabolite concentrations to the amount of tissue is not necessary.
Technical variances and measuring fluctuations occurring from daily technical deviations (e.g. inter-day differences in instrument tuning) as from variations in the reproducibility of the measurement, where normalized be the mean metabolite concentration of 5 homogeneous quality control samples (pooled mouse plasma) between the different Absolute*IDQ*™ p180 kit plates.

2.5.9. Statistical interpretation

No single statistic approach has the power to describe the strength of a relationship between observations. Effect size tells something about the difference in means, but a difference between the samples means occur by chances. Only the combination of effect size, confidence interval or p-value allows the interpreting of the results [125], [126]. Therefore, all three were taken into account in this thesis for interpreting the data.

2.5.10. Statistical limitations and strengths of the animal study

Limitations: The sample number (n=10) was small compared to the number of observed metabolites and metabolite ratios. The study was underpowered to detect smaller effects and a certain number of effects will be missed just be chance. The power of a statistical test describes the probability of the test to do not committing a type II error. Tests with a low statistical power may miss true positive values [127].

Strengths: Even with strict correction for multiple testing, false positive results may occur. With the combination of different statistical methods false positives can be omitted and a justification of the results became more valid. The use of littermate mice and the application of strict parameter for defining a mouse as diabetic and consider the circadian rhythm of the animals should reduce the biological variance to a minimum.

3. Results

Investigation of metabolite homeostasis provides deep insights into the regulatory mechanism behind the changes in biochemical pathways caused by normal or pathological processes, effects of nutrition, environmental impact or pharmacological intervention. However, the specific variations of metabolite levels in peripheral tissues, their biochemical regulations, and especially their interaction have not yet been well examined.
To get deeper insight into metabolite homeostasis in diabetes, I analyzed the metabolic profiles of a diabetic mouse model in response to acute and sub-chronic treatment with antidiabetic drugs and to the pathological state, focusing on systemic and organ specific changes in biochemical pathways.

To investigate in parallel the pharmacokinetics of metformin and its tissues specific distribution, I developed and validated a quantification method for metformin.

3.1. Metformin quantification in mouse tissue and plasma

Metabolomics required a special sample handling and preprocessing of tissues [78], [79]. Therefore, the here developed quantification method should be able to determine the metformin concentration directly from samples which were preprocessed for metabolomics analysis.

3.1.1. Determination of MS/MS parameters

Optimization of ion source and mass spectrometer parameters is necessary to achieve high sensitivity and high selectivity for the analyte of interest. To avoid the detection of artificial peaks two MRMs were recorded for metformin as well as for the internal standard, one MRM for quantification and one as qualifier. In order to determine the optimum conditions for the MRM mode, the ion optic and mass spectrometric parameters were optimized using the automatic compound optimization of the Analyst 1.5.1 software. The optimal MRM parameters were additionally validated manually and presented in Table 3 (chapter 2.4)

3.1.2. Method validation

Calibration curves were constructed by calculating the ratio of metformin to the internal standard (metformin-d6). Linearity of the method was tested with a series of dilutions with 12 steps from 0.39 pmol/ml-1.16 nmol/ml metformin for every tissue homogenate and plasma. Tissue homogenate was prepared as stated in Table 2 and spiked with the accordant metformin concentration. Linear response were obtained in all tissues and plasma with r^2>0.994 in the range from 3.9 pmol/ml-1.16 nmol/ml. The limit of detection (LOD) in tissues was generally better than those observed in plasma. The increased sensitivity is likely due to the fact that tissue homogenate were preprocessed, proteins were precipitated, and interfering substances were diluted. LOD for tissues was found to be below 0.93 pmol/ml, and LOD for plasma was 5.6 pmol/ml. The LOQ for tissues was 3.9 pmol/ml and equal to the lowest point in the linear calibration curve. The LOQ for plasma (5.6 pmol/ml) was equal to the respective LOD.

Intra-day and inter-experimenter precision were determined with two different concentrations 0.5 nmol/ml and 5 nmol/ml. For every concentration and every experimenter 5 aliquots were subjected to the sample preparation procedure. Coefficients of variation (CVs) were calculated and found to be satisfactory within the international accepted acceptance criteria for bioanalytical methods < 15% [128] as presented in Table 4.

Table 4: intra-day and inter-experimenter variability

metformin concentration	intra-day CV	inter-experimenter CV
0.5 nmol/ml	11.10%	12.80%
5 nmol/ml	6.20%	9.90%

3.1.3. Metformin concentrations in tissue and plasma

The metformin concentrations were quantified with the above described method in testis, lung, cerebellum, adipose tissue, liver, kidney and plasma from SGLT2-Inhibitor (SGLT2I), metformin and combination treated mice from the acute (4 h) and sub-chronic (14 day) treatment groups. In SGLT2I treated mice no peaks or only peaks below the LOD were detected.

The metformin concentrations in all samples from the 4 h acute treated animals were clearly higher than in the respective sub-chronic groups (Figure 1). This was caused by the different time points after the last metformin treatment and reflects the known pharmacokinetics of metformin [129].

The metformin concentration in the combination treated groups were found to be significantly lower than in acute and sub-chronic metformin treated groups for adipose tissue, liver, kidney, and plasma. For testis only the sub-chronic combination treated group showed significant lower metformin concentration compared to the only metformin treated group (Figure 1).

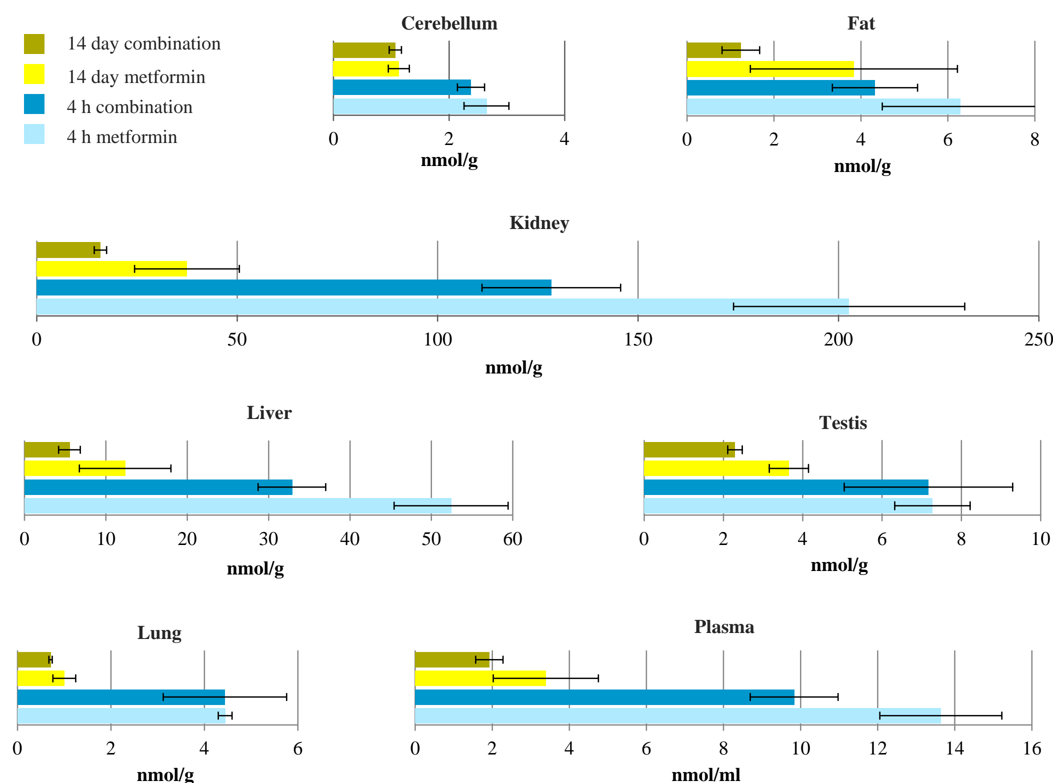


Figure 1: Metformin concentration in mouse tissue and plasma of metformin and combination treated mice from the 4 h and 14 day treatment groups.
In each panel, the treatment regimen and duration is on the y-axis, while mean metformin concentrations ± SEM are shown on the x-axis.

3.2. Glucose regulation by antidiabetic treatment

3.2.1. Systemic glucose regulation in plasma

Antidiabetic therapies were evaluated by their ability to normalize systemic glucose level. The Biocrates Absolute*IDQ*™ p180 kit allows the quantification of metabolites of several substance classes including hexoses. The mainly occurring hexoses in mammals are glucose, galactose, fructose and mannose. The measured hexose concentration is the sum of all occurring hexose molecules in plasma. However, for plasma the concentration of other hexoses than glucose is negligible, and the sum of hexoses reflects mainly the concentration of plasma glucose. According to the Human Metabolome Database (HMDB) [62] the sum of the concentrations of all other hexoses, even in disease states were below 2.5% of the plasma glucose content.

In both groups (acute and sub-chronic), the vehicle treated diabetic phenotype showed a more than 2-fold elevation of plasma hexose levels compared to the wildtype (Figure 2).
During the acute treatment 8 week old mice were treated with a single drug dose 4 hours before the killing of the animals. The acute treatment with the SGLT2I and the combination treatment led to a significant decrease of plasma glucose levels, whereas metformin treatment resulted in an apparent but not significant reduction of plasma glucose levels (Figure 2).
During the sub-chronic treatment, the drug intervention was taking place for 14 days with daily drug administration. The last administration of the drugs was 18 hours before the organ withdrawal. Taking a look at the pharmacokinetics of metformin (t_{max} 2-4 h, $t_{1/2}$ 6-8 h) and the SGLT2I AVE2268 (t_{max} 0.25-0.5 h, $t_{1/2}$ 2-6 h (information from Sanofi-Aventis)), the drugs were mostly excreted by renal clearance at the time of the organ withdrawal. Only the combination treatment significantly reduces the glucose levels in plasma of db/db mice more than 18 hours after the last application. None of the single drugs exhibit such a long-lasting effect on the plasma glucose levels.

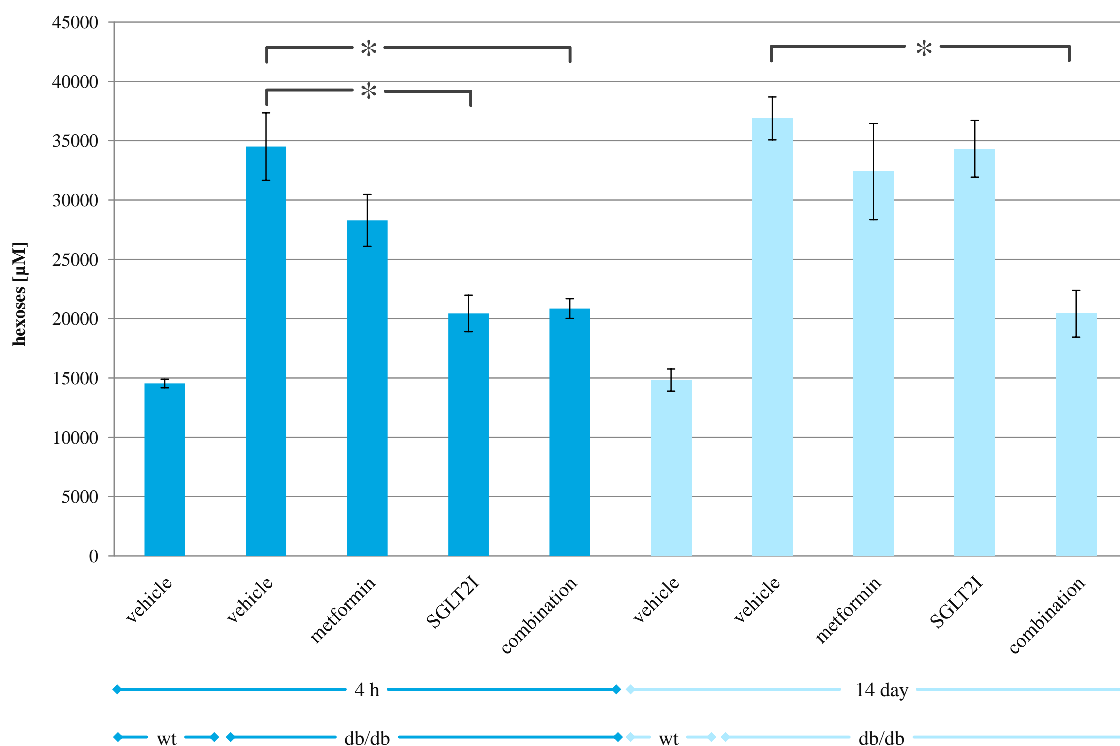


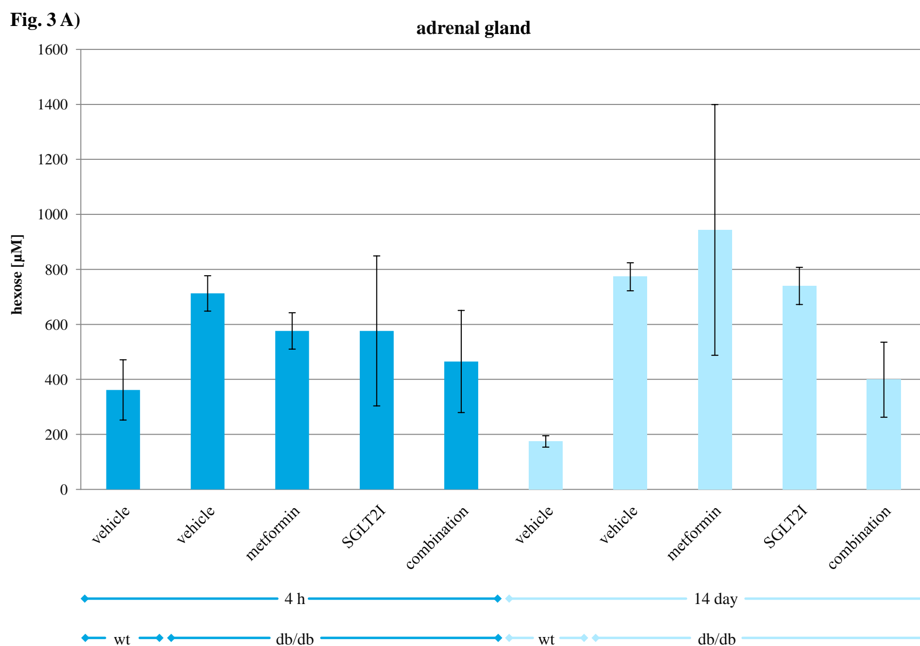
Figure 2: Plasma hexose concentration for diabetic phenotype and after drug treatments. Plasma hexose concentrations in vehicle, metformin, SGLT2I, or combination treated *db/db* and wt mice. The 4 h group was treated with a single drug dose and plasma hexose concentrations were determined 4 h posttreatment. The 14 day group was treated once per day and hexose concentration were determined 18±2 h posttreatment. Statistic significant changes were only highlighted between the db/db animals.

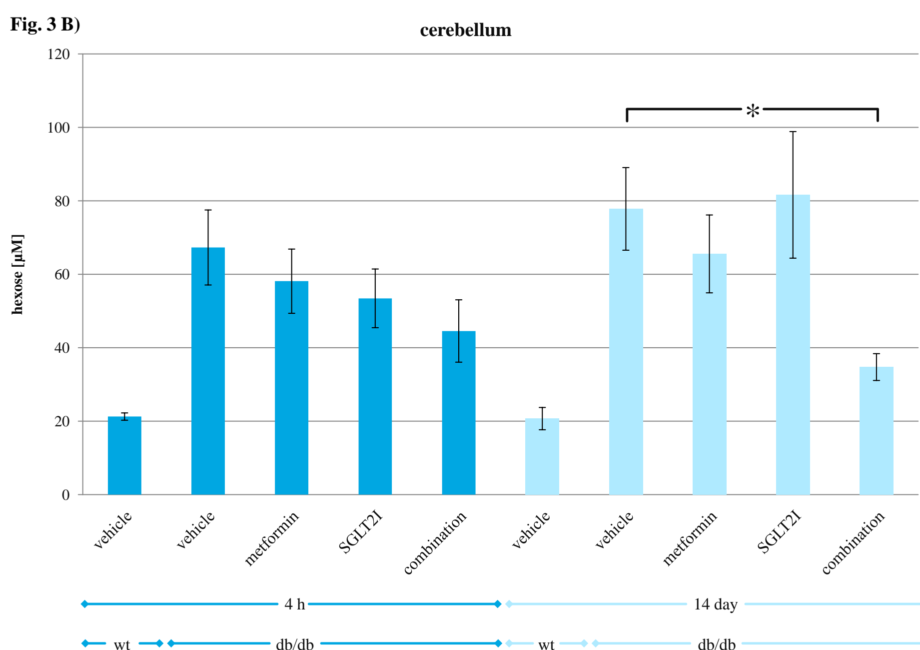
3.2.2. Effect of glucose lowering drugs on tissues

Antidiabetic treatment is often associated with increased insulin sensitivity or enhanced glucose uptake by peripheral tissues. To investigate the tissue specific glucose/hexose levels in response to the antidiabetic treatments, the absolute concentration of the sum of hexoses was quantified using the Biocrates Absolute*IDQ*™ p180 kit. As described in the paragraph above, hexose do not necessarily reflect only glucose but also other hexoses.

The observed concentration changes in peripheral tissues during the antidiabetic treatment (Figure 3 A-E) clearly follow the plasma hexose levels (Figure 2). During the acute treatment only SGLT2I and combination treatment showed clearly lowered hexose levels in the peripheral tissues (Figure 3 A-E). In contrast, liver hexose levels remained constant during the two treatments and only metformin treatment resulted in significantly enhanced hexose levels (Figure 3 F). During the sub-chronic

treatment, only the combination treatment led to significant lowered hexose levels in all tissues (Figure 3 A-F).





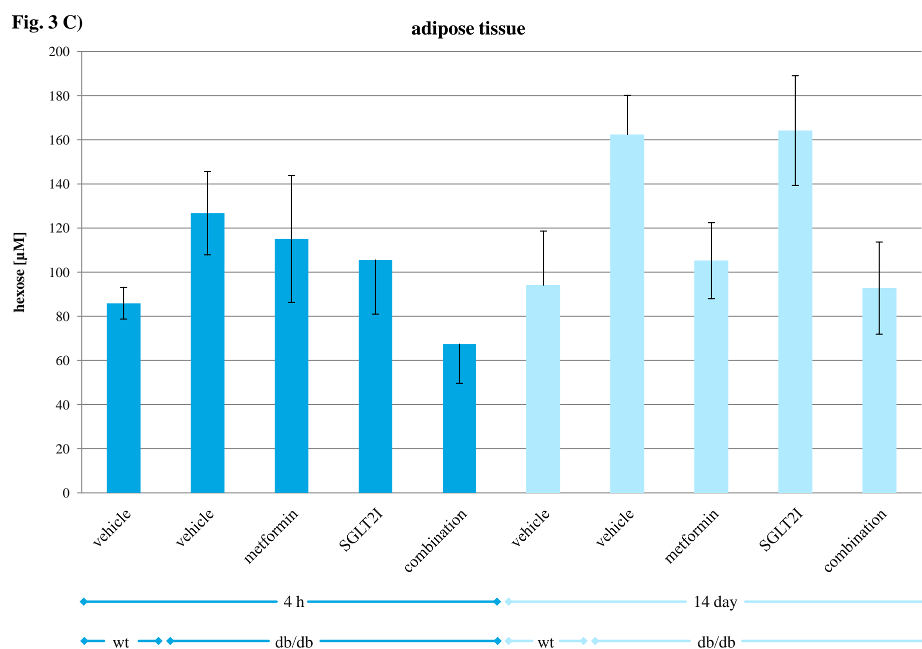


Fig. 3 C)
adipose tissue
hexose [µM]
200
180
160
140
120
100
80
60
40
20
0
vehicle
vehicle
metformin
SGLT2I
combination
vehicle
vehicle
metformin
SGLT2I
combination
4 h
14 day
wt
db/db
wt
db/db

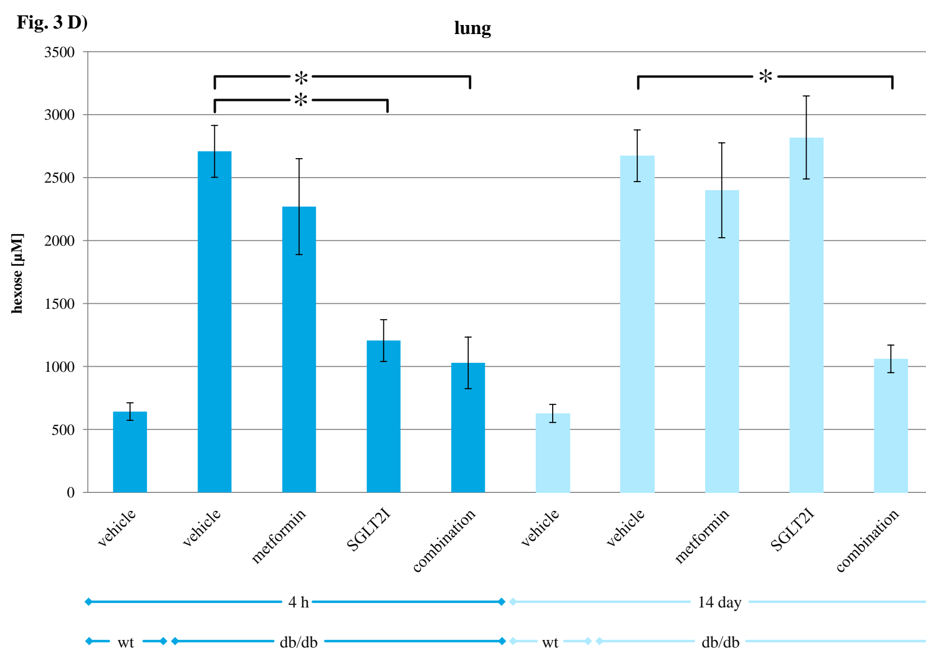


Fig. 3 D)
lung
hexose [µM]
3500
3000
2500
2000
1500
1000
500
0
*
*
*
vehicle
vehicle
metformin
SGLT2I
combination
vehicle
vehicle
metformin
SGLT2I
combination
4 h
14 day
wt
db/db
wt
db/db

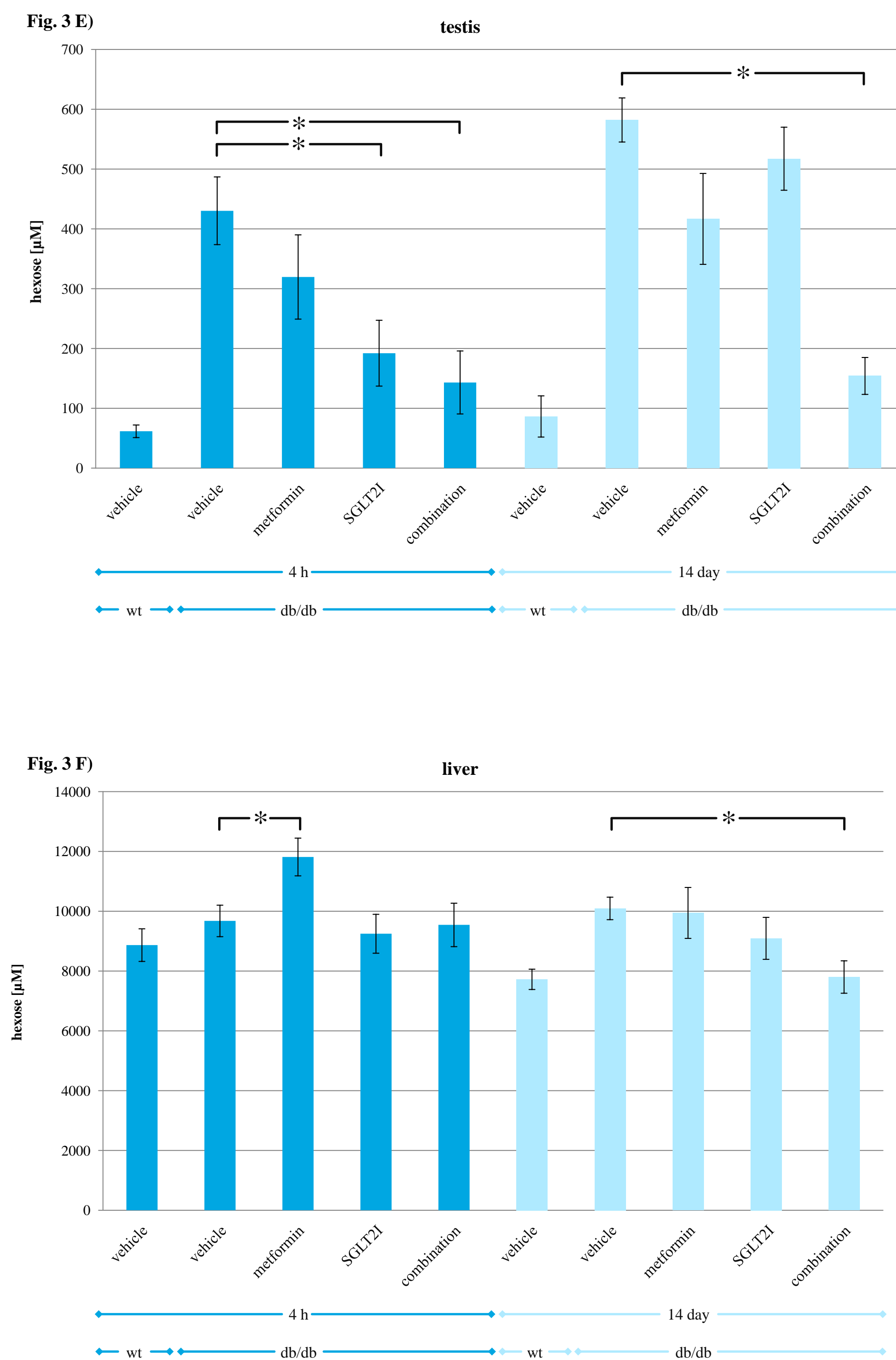


Figure 3: Tissue hexose concentrations for drug treatments and diabetic phenotype
Tissue hexose concentrations in vehicle, metformin, SGLT2I, or combination treated *db/db* and wt mice. The 4 h group was treated with a single drug dose and plasma hexose concentrations were determined 4 h posttreatment. The 14 day group was treated once per day and hexose concentration were determined 18±2 h posttreatment. A) adrenal gland, B) cerebellum, C) adipose tissue, D) lung E) testis, F) liver. Significant changes were indicated for the drug intervention only.

3.3. Metabolic perturbation in diabetic mice induced by drug treatment

The db/db mouse model develops crucial signs of the metabolic syndrome, including obesity [130], elevation of plasma insulin levels, uncontrolled rise of blood sugar levels in combination with impaired glucose tolerance [131], depletion of pancreatic beta-cells [132], increase in gluconeogenic enzyme activity, and reduced insulin sensitivity [133], [134]. This led to systemic as well as site specific alterations in hormonal and metabolic homeostasis. A treatment with antidiabetic drugs should help to reestablishing the normal, healthy metabolic homeostasis in the body.

In this context, the systemic and organ specific response in metabolite homeostasis to acute and sub-chronic pharmacological intervention was studied. The thesis focuses on the effects of the antidiabetic treatment on peripheral organs (testis, lung, adrenal gland, cerebellum) and systemic alteration reflected in plasma. However liver and adipose tissue are primary sites of action for lipid and amino acid metabolism [135]–[137]. To get insight into the site specific origin of the metabolic changes the here presented metabolite profiles were compared to the metabolite profiles of the primary target organs of the metabolic syndrome, liver and adipose tissue. A detailed analysis of changes in liver and adipose tissue was presented by Markus Scheerer [138].

For statistical analysis of the metabolic perturbations, the mice of the vehicle treated diabetic group were used as control group. All drug treated diabetic groups were compared to the vehicle treated diabetic group. CI, q-value, p-gain, and GlassΔ effect sizes of the significant altered metabolites and metabolite pairs were presented in Appendix 6.1.1 and 6.1.2. The tables contain all significant changes in single metabolite levels with q-value<0.05 and metabolite ratios with q-value<0.05 and a p-gain>100. Positive effect size denotes an elevation of metabolite concentration or ratio in the drug treated animals, negative effect size a decrease, respectively. For a detailed description of the applied statistical method refer to the statistic chapter 2.5.
For better visualization of the differences between the groups, and for easier detection of clusters with strong alterations, the GlassΔ effect sizes in the heatmaps (Figure 5 to Figure 18) were only plotted in selected intervals. Values outside of the interval were visualized as minimal or maximal value of the interval. Taking into account, that more than 85% of all effect sizes exhibited absolute values were within the selected intervals, only the detailed information about some rare, very strong alteration will be not be visible in the figures. Metabolites in the heatmaps were sorted together by hierarchical clustering using complete linkage as clustering method.

3.3.1. Comparison of drug induced changes in metabolite profiles

To investigate if the drug induced perturbations were tissue specific or appear on a systemic level, the changes found significant in at least one of the tissues or in plasma were compared to those in the other samples. Even if not significant, these global trends from all groups provide additional information about the origin of systemic and organ specific perturbations and the interplay between the different tissues and plasma, and additionally give insight into site specific drug action.

3.3.1.1. Drug induced changes in amino acid and acylcarnitine profiles

To maintain continuous energy supply considerable amounts of glucose and fatty acids are permanently oxidized. Long chain fatty acids are transported as acylcarnitines into the mitochondrion as well as through other cellular membranes [139]. Therefore, acylcarnitines (AC) contribute to the oxidation of fatty acids. During insulin resistance and T2D the transport and mitochondrial processing of fatty acids are disturbed [139]. In case of insufficient fatty acid or hexose availability amino acis (AA) are used as alternative energy source. The degradation products of leucine, isoleucine, lysine, tryptophan, and valine can be transformed to C3 and C5 acylcarnitines and enter the tricarboxylic acid cycle (Figure 4) [140]. Besides the transport to the mitochondrion, ACs are transported to the blood stream and can be found in plasma [139].
Several studies reported distinct changes in plasma AC profiles of diabetic subjects [83], [141], [142]. Therefore, systemic (plasma) and organ specific alterations in AC and AA homeostasis caused by acute and sub-chronic drug intervention were investigated.

The clustered effect sizes of the 4 h, acute treatment groups give an insight into the fast primary response to the drug intervention (Figure 5). The acute regulation of energy, glucose and fatty acid homeostasis during glucose or energy shortage take place in the liver via liberation of glucose or fatty acids stored in the liver and released into the blood. Indeed, the majority of and most pronounced effects were observed in the liver as well as in plasma. The response to the SGLT2I and combination treatment was more pronounced than in the metformin treated groups. Treatments including the SGLT2I led to an immediate and strong reduction of blood glucose levels, whereas metformin act by manipulating the energy homeostasis on the cellular level and response was more slowly.

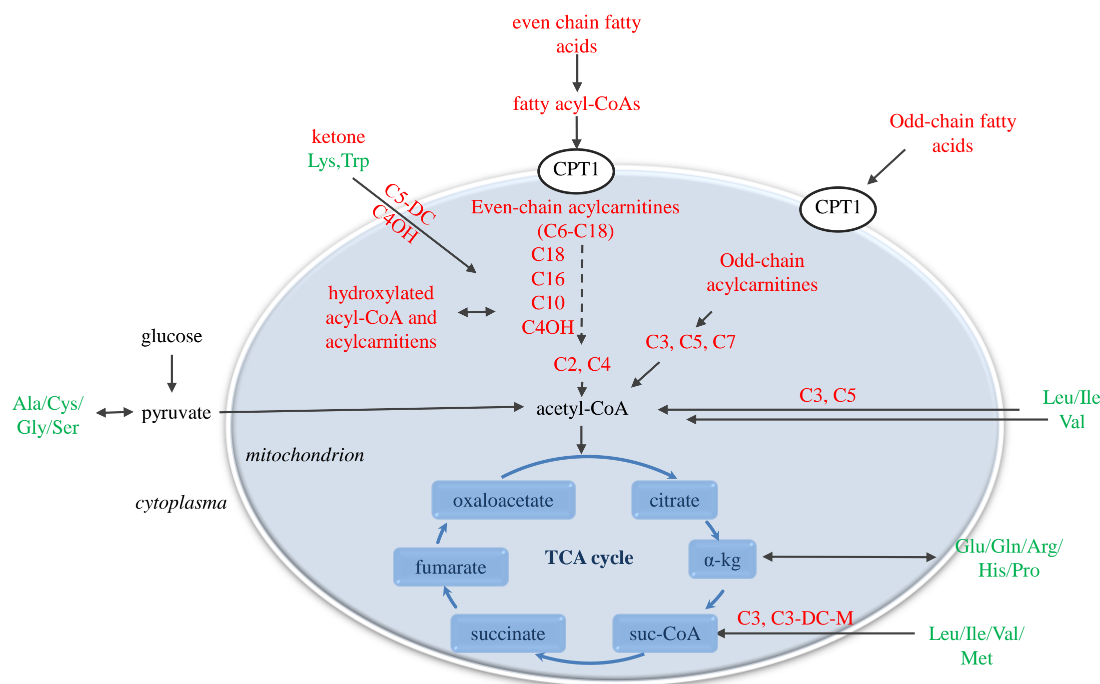


Figure 4: Generalized scheme for pathways of tricarboxylic acid cycle, fatty acid oxidation, and amino acid catabolism (adapted from [143]).
During normal energy state, pyruvate derived from glucose is the main source of energy. In case of lack of glucose, pyruvate is used for gluconeogenesis. Long-chain fatty acids, amino acids (mainly branched chain AAs), and ketones are used as alternative substrates for acetyl-CoA syntheses. Fatty acyl CoAs crosses the mitochondrial membranes as acylcarnitines.CPT1, carnitine palmitoyltransferase; TCA, tricarboxylic acid cycle; α-kg, alpha ketoglutarate; suc-CoA, succinyl CoA.

Liver and plasma metabolite concentrations were found to be inversely regulated for some metabolite groups during the acute intervention. The massive elevation of long-chain ACs in plasma of SGLT2I treated mice was accompanied by depletion of long-chain ACs in the liver. On the other side free carnitine (C0) and short-chain AC were depleted in plasma and enriched in liver.
The majority of the amino acids, which were depleted in plasma, were found to be elevated in the liver with two exceptions. The levels of the BCAA Ile and Leu were elevated in both, liver and plasma, pointing to the special behavior of BCAAs during the acute SGLT2I intervention.

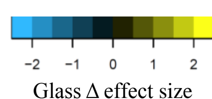
A global response to the acute treatment with SGLT2I, and in part to the combination treatment appeared in nearly all organs for Ile (which was found significantly increased during the SGLT2I treatment) as well as for the ratios of Ile to Met, Lys, Thr, Trp, Phe, Tyr, Pro.
Additionally, C4-OH(C3-DC) (3-hydroxy-butyrylcarnitine) and its ratio to Lys or Trp were found elevated in all investigated organs, although it did not reach the significance level (data not shown).

C4-OH is one of the products of Lys and Trp degradation and can be converted to acetyl-CoA (Figure 4) [91]. Additionally, the concentration of Ala, a marker for short-term metabolic changes [144] was decreased in plasma.

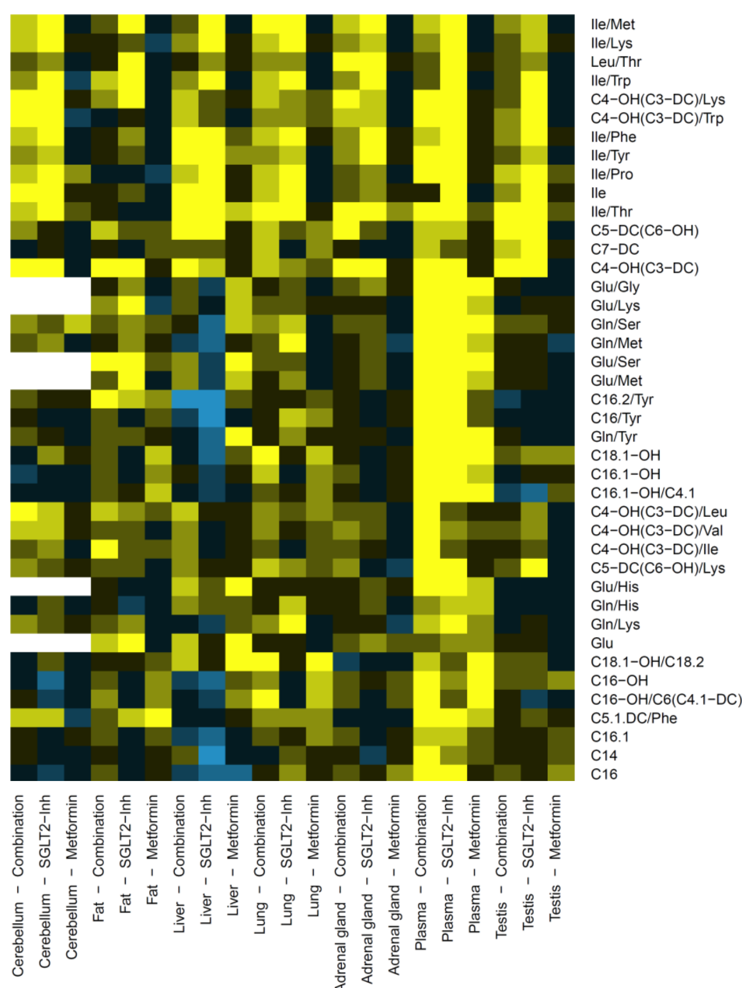
Further, ratios of C4-OH(C3-DC) and C5-DC(C6-OH) (glutaryl-carnitine) to several metabolites from all classes were significantly changed, together with high p-gains in all tissues and plasma during SGLT2I and combination treatment (Appendix 6.1.1). Taking into consideration, the significantly increased concentrations of the single metabolites, this indicates a huge impact of these two treatments on either hydroxyl acylcarnitine or dicarboxy acylcarnitine metabolism. Because of the immense number of ratios containing theses acylcarnitines, they were not presented in Figure 5 (only in Appendix 6.1.1).

Next to plasma and liver, also metabolites in the lung exhibit a particular response to the SGLT2I and stronger to the combination treatment. In lung and plasma of the combination treatment groups, elevated levels of long-chain (C14-18) hydroxylated ACs were observed. This indicated a liver independent, tissue specific, response to the acute treatment (Figure 5).

Figure 5



Glass Δ effect size



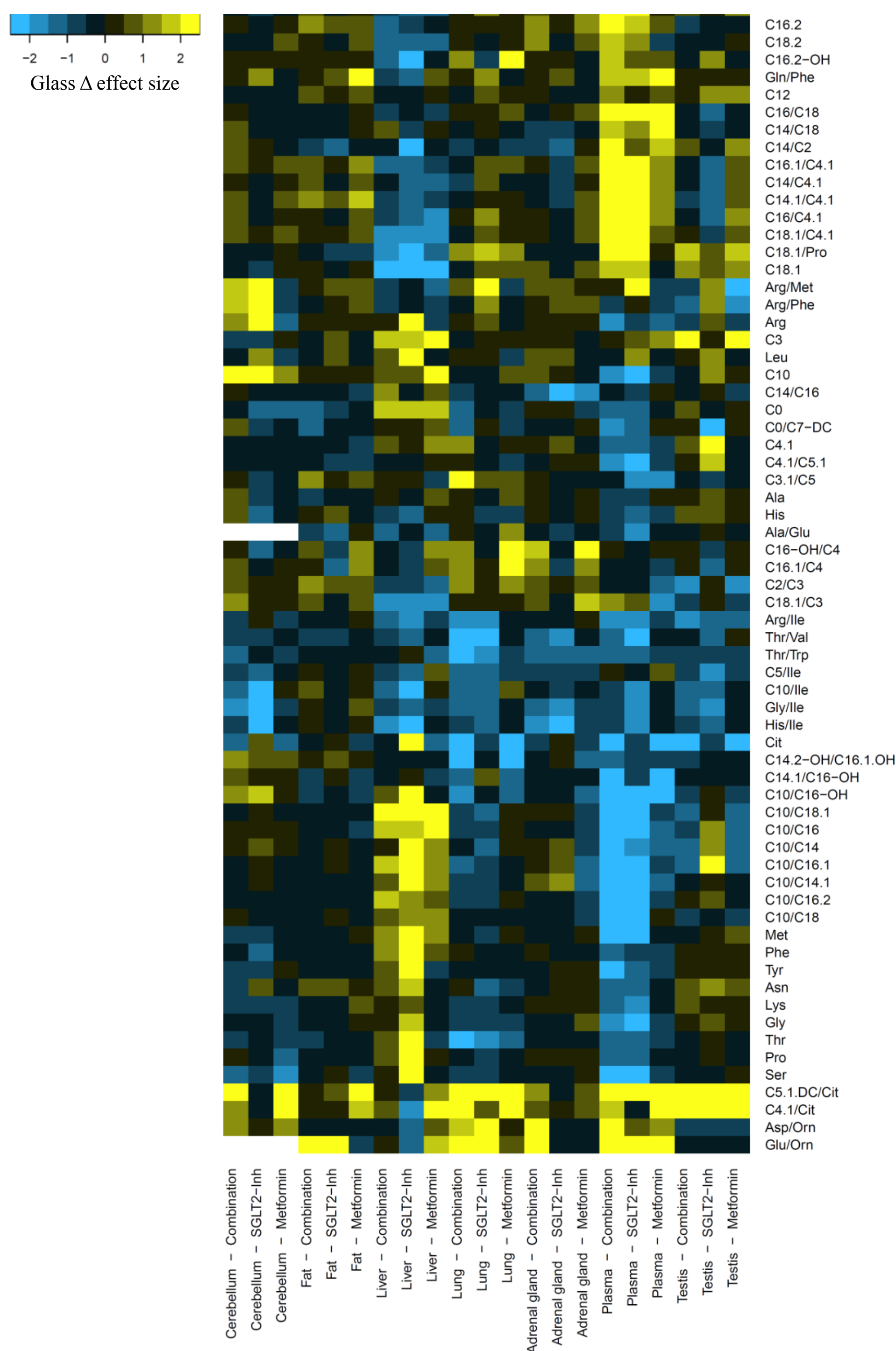


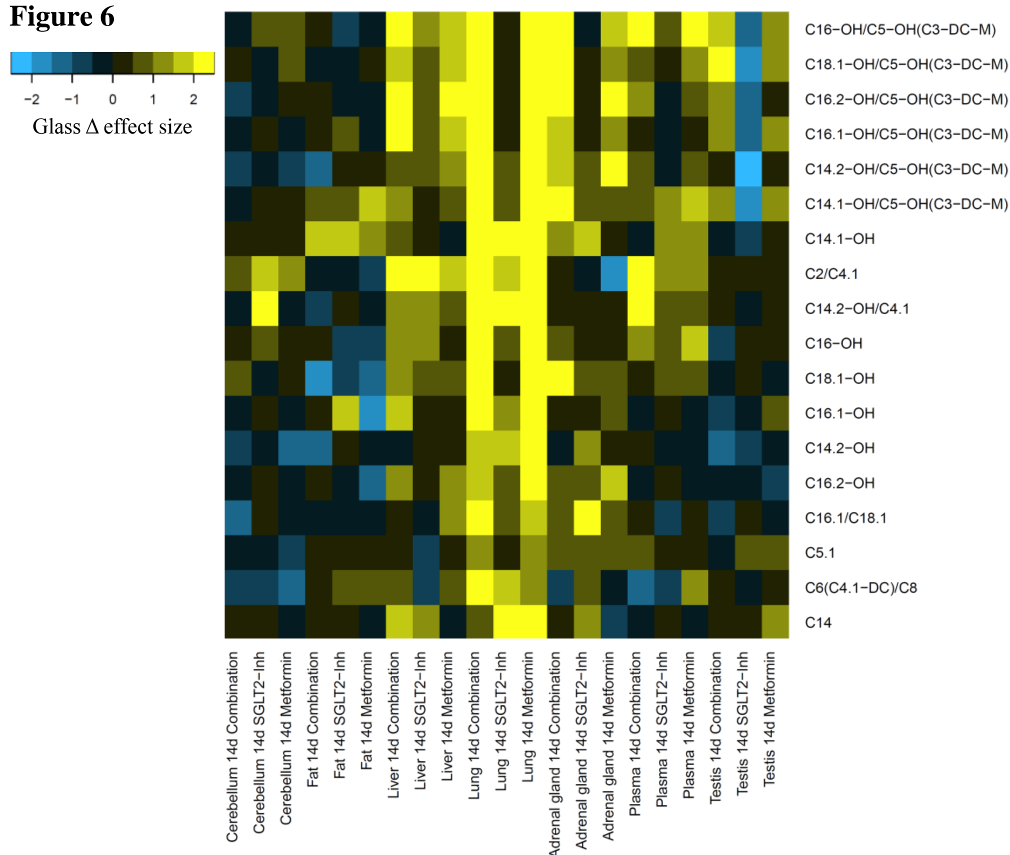
Figure 5: Drug induced differences in amino acid and acylcarnitine levels during acute treatment. For acute treatment, 8 week old db/db mice were treated with a single dose of drug or vehicle. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the treatment groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

In contrast to the fast response of the organism to the acute treatment the sub-chronic intervention represent the more long lasting effects. While in the acute treatment the effects of the rapid glucose lowering through the SGLT2I were most severe, only minor effects of the single SGLT2I administration were observed during the sub-chronic treatment. None of these alterations were found to be significant (Appendix 6.1.2).

The metformin and combination treatment groups of the tissues exhibited similar regulation pattern to each other. This pointed to a close similarity in the response between metformin and combination treatment for all tissues. The most prominent changes for metformin and combination treatment were found for the AC and AA metabolism and the AC/AA ratios.

Taking a look at the effects in the sub-chronic groups, the strongest effects appeared in the lung of metformin and combination treated mice. In both treatment groups increased hydroxylated acylcarnitines (AC(OH)) and increased ratios of theses to C5-OH(C3-DC) were observed. This indicated an increase in fatty acid hydroxylation. Additionally, alterations in the ratios of C5-OH(C3-DC) to AA (Lys, Trp, Val, Ile, Phe) were observed (Figure 6, Appendix 6.1.2), pointing to changes in AA degradation.

In contrast to the metformin treatment in which one AC was found significantly up-regulated, the combination treatment resulted in significant decrease of plasma AC levels (Appendix 6.1.2).



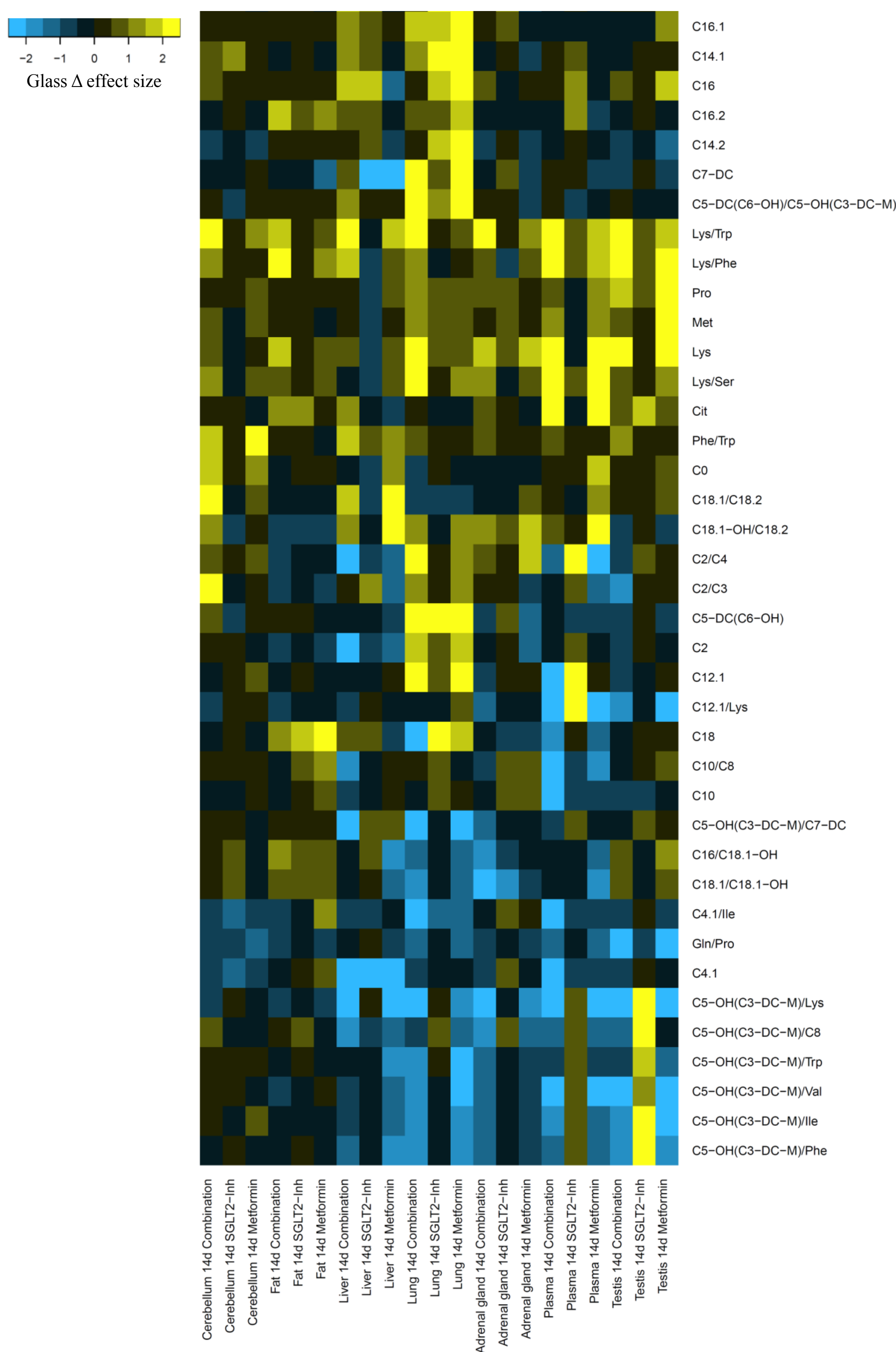


Figure 6: Drug induced differences in amino acid and acylcarnitine levels during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the treatment groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

Interestingly, the massive alteration in BCAA levels during the acute treatment was not followed by any response during the sub-chronic treatments. One cause for this observation was probably the differences between the 8 week and 10 week diabetic phenotype. However, as shown in Table 5, the db/db phenotype had indeed a significant impact on BCAA levels on both time points. Plasma levels of all three BCAA were significantly increased in the db/db mouse model. Additionally, the tissue levels indicate generally an increase of BCAA levels in db/db mice. However, not all of them reached the significant level.

Table 5: Alteration in BCAA levels between wildtype and diabetic mice.
q-values and effect sizes for changes in BCAA (Ile, Leu, Val) concentration between vehicle treated wild-type and db/db mice in the acute (4 h) and sub-chronic (14 day) groups. Negative effect sizes indicated a decrease of metabolite levels in the wildtype compared to db/db mice.

		Cerebellum		Lung		Plasma	
metabolite		*q-value*	*effect size*	*q-value*	*effect size*	*q-value*	*effect size*
Ile	4 h	0.0007	-1.98	0.0944	-0.42	0.000096	-1.57
Leu	4 h	0.0110	-1.08	0.0608	-0.51	0.010403	-0.92
Val	4 h	0.0232	-0.98	0.0809	-0.42	0.005963	-0.86
Ile	14d	0.0021	-1.72	0.0176	-0.74	0.000001	-2.13
Leu	14d	0.0017	-2.00	0.0187	-0.80	0.000015	-1.49
Val	14d	0.1024	-0.82	0.0581	-0.56	0.000075	-1.53

		Adrenal gland		Testis	
metabolite		*q-value*	*effect size*	*q-value*	*effect size*
Ile	4 h	0.3244	-0.51	0.4386	-0.41
Leu	4 h	0.3426	-0.53	0.4151	-0.51
Val	4 h	0.4131	-0.44	0.2381	-0.70
Ile	14d	0.0023	-1.47	0.0047	-1.39
Leu	14d	0.0098	-1.14	0.0921	-0.69
Val	14d	0.0093	-1.28	0.0199	-0.96

3.3.1.2. Changes in citrulline levels in response to antidiabetic treatment

Diabetes and the metabolic syndrome are closely related to cardiovascular diseases, endothelia dysfunction, and hypertension. The negative impact of endothelia dysfunction in diabetes results in high blood pressure and increases the risk of ischemia, kidney failure, and blood vessel damage [145].

The main regulator of vascular tone is nitric oxide which is produced in the vascular endothelium by the conversion of arginine (Arg) to citrulline (Cit) and nitric oxide via the endothelia nitric oxide synthase (eNOS).

There are two biological pathways which are involved in Arg recycling from Cit. One is the nitric oxide cycle, appearing in kidney and the vascular system. The other pathway is the urea cycle taking place mainly in liver (Figure 19).

During the data analysis of the metabolomics experiments Cit was found to be significantly altered in nearly all sample groups of the acute drug treatment and also in plasma of the combination treated animals in the sub-chronic group. The acute response to the drug intervention was a depletion of Cit in lung, testis, and plasma, and an increase in liver (Figure 7 A) which where correlated with increased Orn levels. If the treatment was continued for 14 days, organ specific Cit levels returned to the level before the drug intervention. Only the plasma Cit concentration was significantly enhanced after the 14 day drug intervention (Figure 7 B).

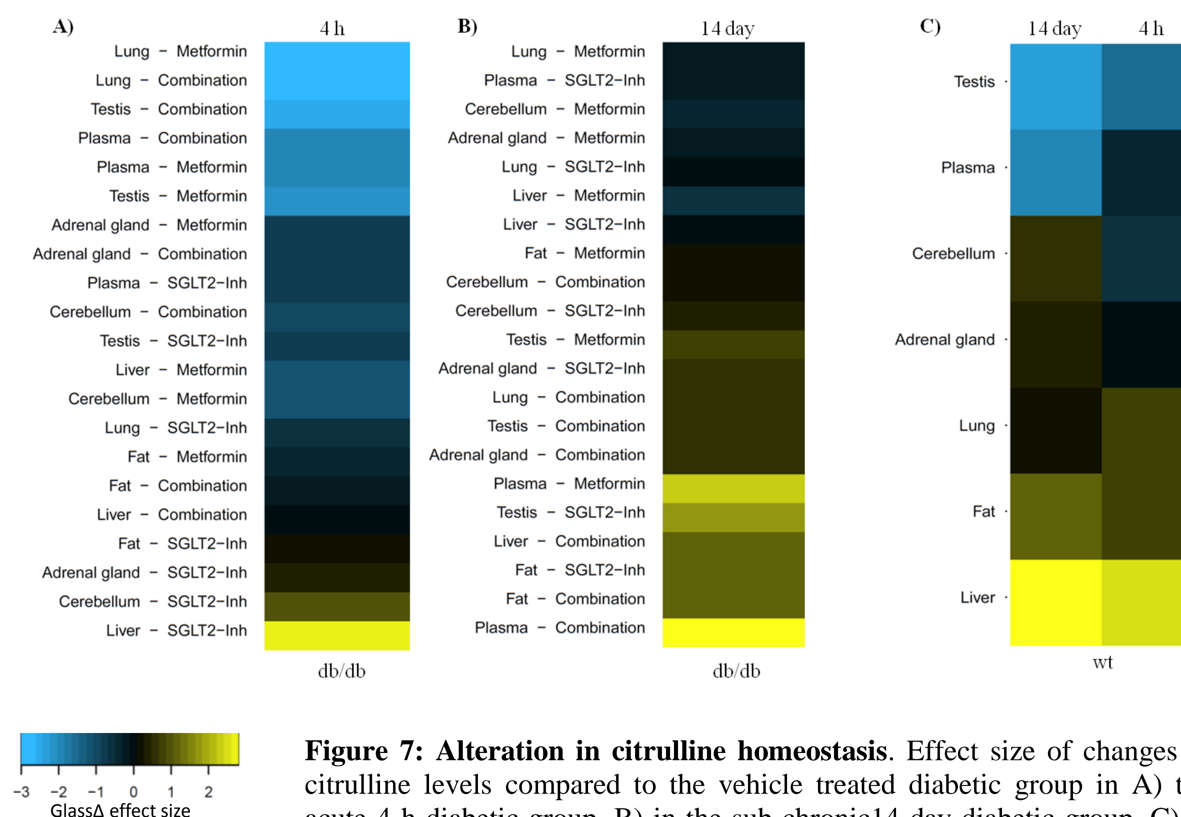


Figure 7: Alteration in citrulline homeostasis. Effect size of changes in citrulline levels compared to the vehicle treated diabetic group in A) the acute 4 h diabetic group, B) in the sub-chronic14 day diabetic group. C) in the wildtype (14 day: 10 week old mice, 4 h: 8 week old mice). Positive effect sizes indicated an elevation in the drug treated or in the wildtype groups, respectively. Effect sizes exceeding 3.0 were set to 3.0 or -3.0, respectively.

Plasma Cit levels in the wildtype mice were found nearly equal to that observed in db/db mice. The same is true for the organ specific Cit levels between wildtyp and db/db mice. Only liver Cit levels were enhanced in the wildtype. The changes of Cit concentration during the combination treatment were much stronger than the alteration between wildtype and diabetic mice (Figure 7, Appendix 6.1). This indicates a very strong response to the combination treatment, which exceed the wildtype level.

3.3.1.3. Drug induced changes in lyso-/phosphatidylcholine profiles

Lipids in general have different biological functions. They act as signal molecules, energy storage and structural components of the cell membranes [146]. The lipid class of phosphatidylcholine (PC) is most abundant in lipoprotein particles in the blood, critical constituents of lung surfactant and makes up a major part of the outer leaflet of cellular membranes. As cellular membranes segregate extra- and intracellular environment, and form organelles within the cell, the lipid composition of these membranes influences the incorporation of proteins (e.g. transfer channels or receptor molecules). Cellular function like transport, cell signaling, fusion and interaction are affected by membrane composition. Changes in cellular PC content alter membrane composition and influence all of these cellular mechanisms. Therefore, the investigation of synthesis and turnover pathways of lipids is crucial for the understanding of pathological phenotypes associated with lipid disorders. The biochemical syntheses of PCs occur in the cytosol of nearly all cells and tissues via the Kennedy pathway [147]. Only in liver, a notable amount of PC is also generated from phosphatidylethanolamines by phosphatidylethanolamine N-methyltransferase (PEMT) [148]. The basic components for the synthesis are CDP-choline and either diacylglycerol or alkyl-acylglycerol resulting in two types of PCs, diacylglycerophosphocholine (PCaa) with two fatty acids bound by ester bonds and acylalkenylglycerophosphocholine (PCae) with one of the fatty acid bound by an ester and one by an ether bound. The fatty acid chains vary in length and position of double bounds, making up a diverse spectrum of phosphatidylcholines. Most commonly in mammals C16:0, C18:0, or C18:1 fatty acid are found as ether bound at the sn-1 position, PUFA are found on an ester linkage at the sn-2 position of the glycerin backbone [149].

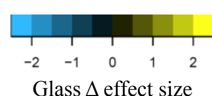
Phosphatidylcholines (PCs) and lysophosphtidylcholines (lysoPCs) levels reflect the fatty acid turnover and the availability or depletion of saturated and unsaturated fatty acids as well as changes in their chain length. It was previously described that under pathological conditions, as diabetes, the composition of the PC and lysoPC profile [83], [84], [150] in liver and plasma were changed in terms of chain length and saturation status. Therefore, changes in PC and lysoPC profiles were investigated.

In response to the acute treatment liver and plasma were the sides of action, whereas only minor changes appeared in lung and adipose tissue. The most severe effects appeared in SGLT2I and combination treated mice, and only in part in the metformin treated groups (Figure 8). The effects observed during the combination treatment mostly reflecting the changes caused by the SGLT2I. The acute antidiabetic treatment with metformin had only minor effects on lysoPC levels. SGLT2I and combination treated mice displayed lower levels of lysoPCs mainly in plasma, and in part in testis and lung.

To investigate if the changes in plasma are caused by changes in lipid homeostasis in the liver during acute drug intervention, the metabolite alterations between plasma and liver were compared. Depletion of metabolites in liver and enrichment in plasma were found for ratios with PCaa32:0 to more saturated and longer PCs (aa32:2, aa34:4, aa36:5, ae36:2) and to lysoPCs (16:0, 17:0, 18:0) indicated a depletion of PCaa32:0 in liver and an enrichment in plasma under SGLT2I and combination treatment. In response to SGLT2I and combination treatment lysoPC (C16:0,C17:0,C18:0) ratios to PCae (34:1, 34:2, 38:5, 36:3, 36:4, 38:0, 38:5, 38:6) were decreased in plasma with only minor changes in the liver.

LysoPC(C16:1) ratios to PCs showed a fast response to metformin treatment in plasma and liver. In both cases the ratios were found to be elevated. In contrast, the SGLT2I and combination treatment induced a decrease in this ratio in plasma and only week response in the liver. A liver specific reaction to all three treatments was observed for lysoPC(18:1) and lysoPC(18:2). Both metabolite concentrations were found to be elevated in response to the 4 h treatments.

Figure 8



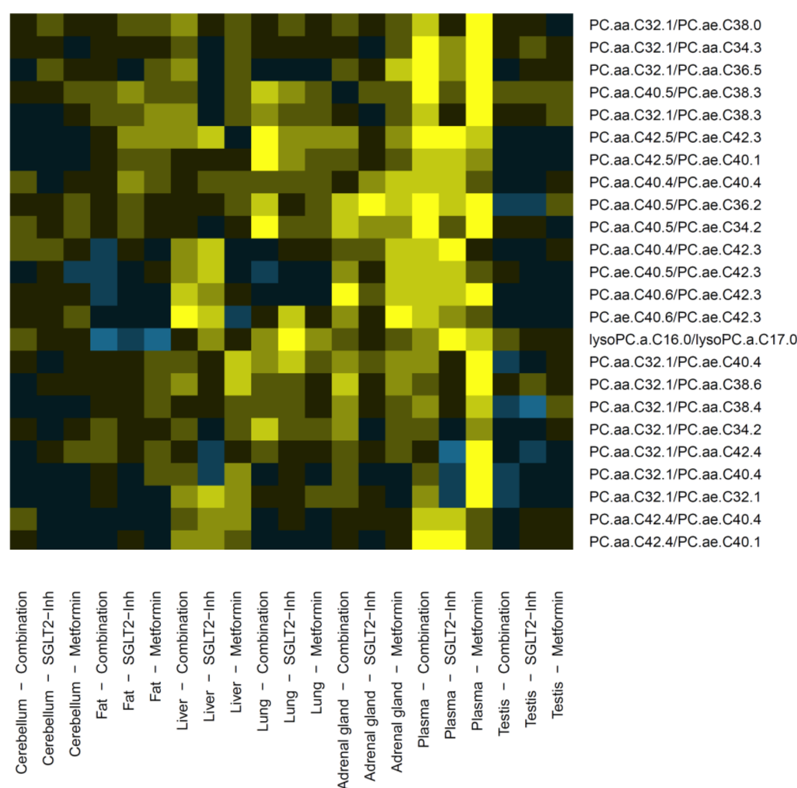


Figure 8

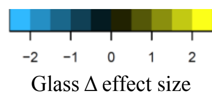
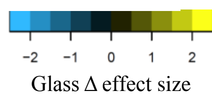


Figure 8



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Cerebellum – Combination
Cerebellum – SGLT2–Inh
Cerebellum – Metformin
Fat – Combination
Fat – SGLT2–Inh
Fat – Metformin
Liver – Combination
Liver – SGLT2–Inh
Liver – Metformin
Lung – Combination
Lung – SGLT2–Inh
Lung – Metformin
Adrenal gland – Combination
Adrenal gland – SGLT2–Inh
Adrenal gland – Metformin
Plasma – Combination
Plasma – SGLT2–Inh
Plasma – Metformin
Testis – Combination
Testis – SGLT2–Inh
Testis – Metformin

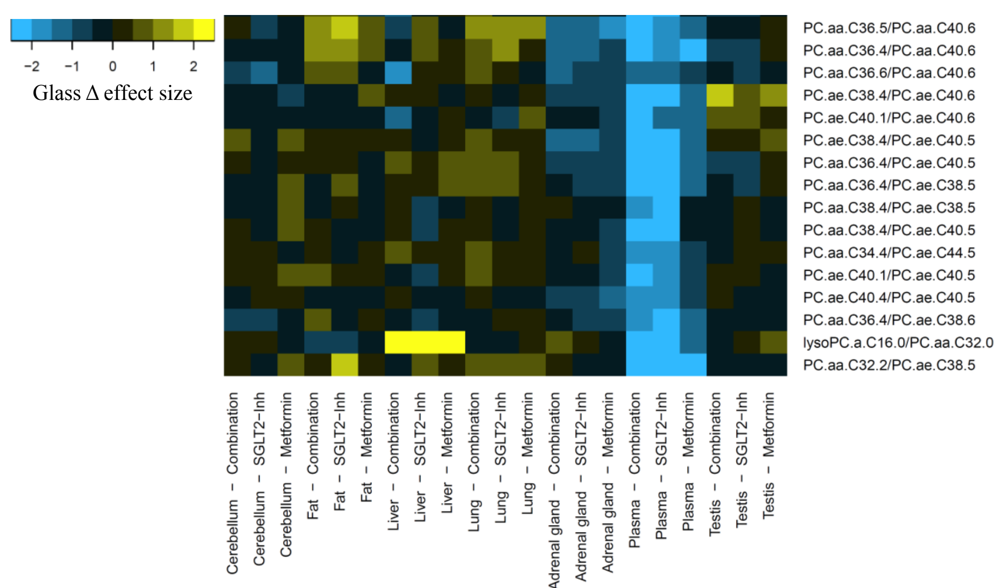


Figure 8: Drug induced differences in phosphatidylcholine and lysophosphatidylcholine levels during acute treatment. For acute treatment, 8 week old db/db mice were treated with a single dose of drug or vehicle. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the treatment groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

Investigating the alteration caused by the sub-chronic treatment, the strongest effects in all organs and plasma appeared during the combination treatment followed by the metformin treatment. The SGLT2I treatment had no significant effect on the observed metabolite levels. Plasma, liver, lung and cerebellum were the most effected sites of action, testis showed only minor changes. The differences in the lipids during metformin treatment are limited to a few PC/PC ratios in lung (10), cerebellum (4), and plasma (2) and to 3 changes in single PCs in plasma (PCaa32.1, PCae42.4, PCae44.4). The PC/PC ratios in lung and cerebellum indicated a shift to more unsaturated and longer chain length PCs. The data from the combination treatment show the same trend in PC/PC ratios to polyunsaturated and longer chain PCs in lung (45 PC/PC ratios, 37 ratios with increased unsaturation, 24 with increased chain length) and in cerebellum (25 PC/PC ratios, 18 ratios with increased unsaturation, 23 with increased chain length) with some special exceptions. In lung the 4 significant changed ratios containing PCae32:2 indicated a shift to shorter chain length and the 2 ratios containing PCae38.0 indicated a shift to the more saturated PC. In cerebellum one ratio containing PCaaC38:0 also showed a decreased number of double bounds. In plasma the same trend to polyunsaturated, long chain PCs was observed for the 113 significantly altered PC/PC ratios (49 ratios indicated increased unsaturation, 58 increased chain lengths).

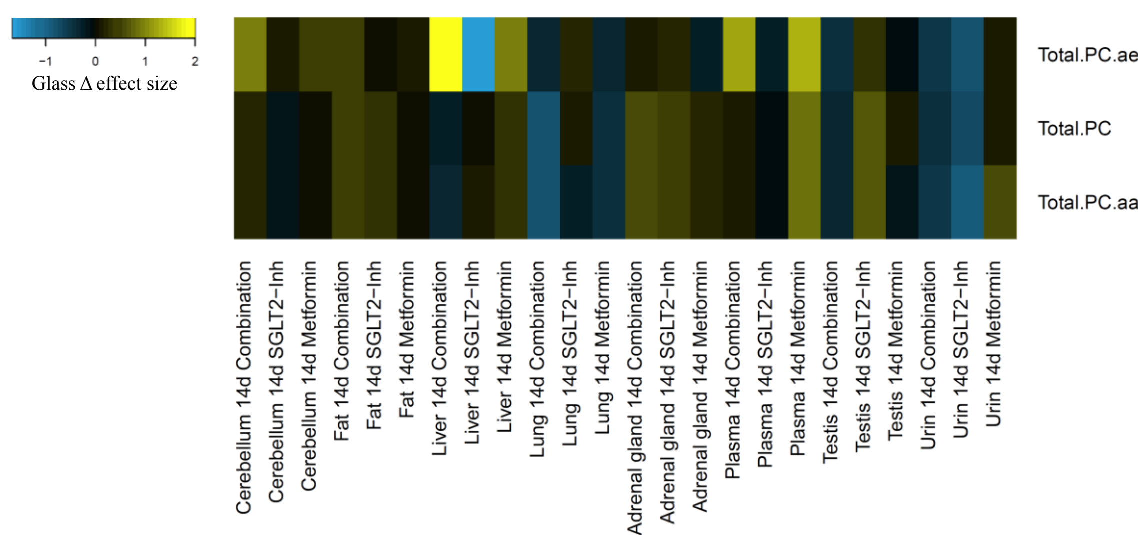


Figure 9: Glass delta effect sizes of total PC, total PCaa, and total PCae during sub-chronic antidiabetic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Effect sizes exceeding 2.0 were set to 2.0 or -2.0, respectively.

The observed shift in PC/PC ratios during combination treatment contained numerous ratios between PCaa and PCae. In lung, 26 of 45 ratios between PCaa and PCae, in cerebellum 11 of 25, and in plasma 69 of 113 ratios were found to be altered. Alteration in the amount of PCaa and PCae can result from enhanced de novo synthesis or from changes in the conversion of lipids. The total amount of PC was not significantly changed in any organ or in plasma (Figure 9). Therefore, the changes may have occurred from alteration in the activity of Lands cycle enzymes (phospholipase A2 (PLA2), lysophosphatidylcholine acyltransferases) or from the phosphatidylethanolamine methyl transferase pathway (only in liver). Significant changes in the ratio of total PCaa/total PCae were only observed in lung, plasma, and liver of combination treated mice. Whereas, the amount of PCaa or PCae alone do not changed significantly (Table 6).

Even if not significant, the trend in lung indicated a decrease in total PC, total PCaa, and total PCae, whereas the trend in plasma indicated an enhancement for these three metabolite groups. This finding was found coherent with the significant changes for single PCs. In lung, three PCaa were found to be significantly downregulated (Table 7). For cerebellum and plasma, all single PCs showed an enhanced concentration during combination treatment (Table 7).

Table 6: Changes in total PC, total PCaa, and total PCae content during combination treatment in lung, plasma and liver

	Lung		Plasma		Liver	
	q-value	effect size	q-value	effect size	q-value	effect size
Total.PC	0.1042	-0.80	0.2185	0.51	0.4395	-0.30
Total.PC.aa/Total.PC.ae	0.0046	1.77	0.0002	2.21	0.0024	1.78
Total.PC.aa	0.0986	-0.82	0.2305	0.48	0.4238	-0.32
Total.PC.ae	0.2403	-0.36	0.0239	1.84	0.0766	2.19

Table 7: Significant changed PC during combination treatment

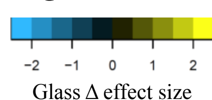
	metabolite	q-value	effect size
lung	PC.aa.C34.2	0.0262	-1.43
lung	PC.aa.C34.3	0.0456	-1.08
lung	PC.aa.C36.3	0.0200	-1.46
cerebellum	PC.aa.C36.5	0.0346	1.29
cerebellum	PC.aa.C36.6	0.0107	1.63
cerebellum	PC.aa.C38.5	0.0390	1.25
cerebellum	PC.ae.C38.0	0.0098	1.8
cerebellum	PC.ae.C42.3	0.0147	1.54
cerebellum	PC.ae.C44.3	0.0252	1.54
cerebellum	PC.ae.C44.4	0.0228	1.7
cerebellum	PC.ae.C44.5	0.0217	1.51

	metabolite	q-value	effect size
plasma	PC.aa.C32.0	0.0375	1.22
plasma	PC.aa.C36.1	0.0234	1.42
plasma	PC.aa.C40.2	0.0449	2.55
plasma	PC.aa.C42.2	0.0275	1.78
plasma	PC.ae.C34.0	0.0254	1.3
plasma	PC.ae.C34.2	0.0140	1.54
plasma	PC.ae.C34.3	0.0168	2.43
plasma	PC.ae.C36.0	0.0114	1.61
plasma	PC.ae.C36.1	0.0271	1.7
plasma	PC.ae.C36.2	0.0135	1.65
plasma	PC.ae.C36.4	0.0379	1.29
plasma	PC.ae.C38.1	0.0056	2.22
plasma	PC.ae.C38.2	0.0005	3.28
plasma	PC.ae.C38.6	0.0310	1.66
plasma	PC.ae.C40.2	0.0215	2.62
plasma	PC.ae.C40.4	0.0146	2.24
plasma	PC.ae.C42.3	0.0197	2.32
plasma	PC.ae.C42.4	0.0074	4.31
plasma	PC.ae.C42.5	0.0227	2.88
plasma	PC.ae.C44.4	0.0087	2.69
plasma	PC.ae.C44.6	0.0276	1.57

Two other observations indicated that the alteration in sub-chronic combination treatment occurred by changes in the Lands cycle, or in another way related to PLA2 activity. The first observation was the increased level of lysoPCs, the second the alteration in the lysoPC/PC-ratios. LysoPCs were found to be enhanced in lung, cerebellum and plasma. The lysoPCs lysoPC(18.1) and lysoPC(18.2) were elevated in plasma, lung and cerebellum, lysoPC(16.0) and lysoPC(20.4) only in lung and cerebellum and lysoPC(17.0) were lung specific, lysoPC(16.1) specific for cerebellum. Alteration in lysoPC/PC ratios were almost exclusively observed in plasma (12 lysoPC/PC ratios).

Summarizing the sub-chronic effects of the administered antidiabetica, all of the SGLT2I treated groups showed similar regulation pattern with no significant response to this treatment for the metabolite class of PCs and lysoPCs. Generally, the SGLT2I treatment seemed not to affect the PC or lysoPC homeostasis in a severe way (Figure 10). The remaining metformin and combination treatment groups showed similar regulation pattern for most of the investigated metabolites and metabolite ratios with clearly stronger and more significant effects during combination treatment with some effects which are unique for the combination treatment (Figure 10).

Figure 10



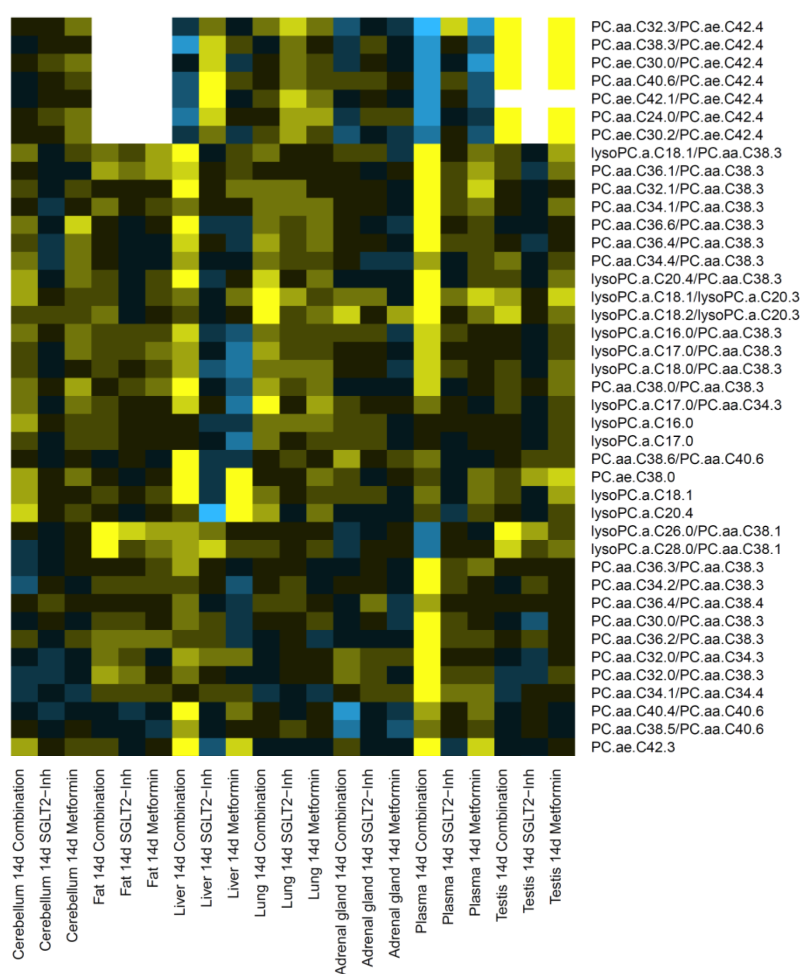
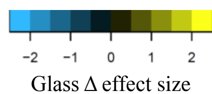


Figure 10



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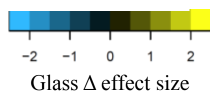
Cerebellum 14d Combination
Cerebellum 14d SGLT2−Inh
Cerebellum 14d Metformin
Fat 14d Combination
Fat 14d SGLT2−Inh
Fat 14d Metformin
Liver 14d Combination
Liver 14d SGLT2−Inh
Liver 14d Metformin
Lung 14d Combination
Lung 14d SGLT2−Inh
Lung 14d Metformin
Adrenal gland 14d Combination
Adrenal gland 14d SGLT2−Inh
Adrenal gland 14d Metformin
Plasma 14d Combination
Plasma 14d SGLT2−Inh
Plasma 14d Metformin
Testis 14d Combination
Testis 14d SGLT2−Inh
Testis 14d Metformin

Figure 10



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PC.aa.C38.3/PC.aa.C42.2
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PC.aa.C34.3/PC.ae.C44.5
PC.aa.C38.3/PC.ae.C44.4
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PC.aa.C38.3/PC.ae.C34.2
PC.aa.C38.3/PC.ae.C36.2
PC.aa.C38.4/PC.aa.C40.4
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PC.aa.C38.3/PC.aa.C40.5

Cerebellum 14d Combination
Cerebellum 14d SGLT2−Inh
Cerebellum 14d Metformin
Fat 14d Combination
Fat 14d SGLT2−Inh
Fat 14d Metformin
Liver 14d Combination
Liver 14d SGLT2−Inh
Liver 14d Metformin
Lung 14d Combination
Lung 14d SGLT2−Inh
Lung 14d Metformin
Adrenal gland 14d Combination
Adrenal gland 14d SGLT2−Inh
Adrenal gland 14d Metformin
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Testis 14d Combination
Testis 14d SGLT2−Inh
Testis 14d Metformin

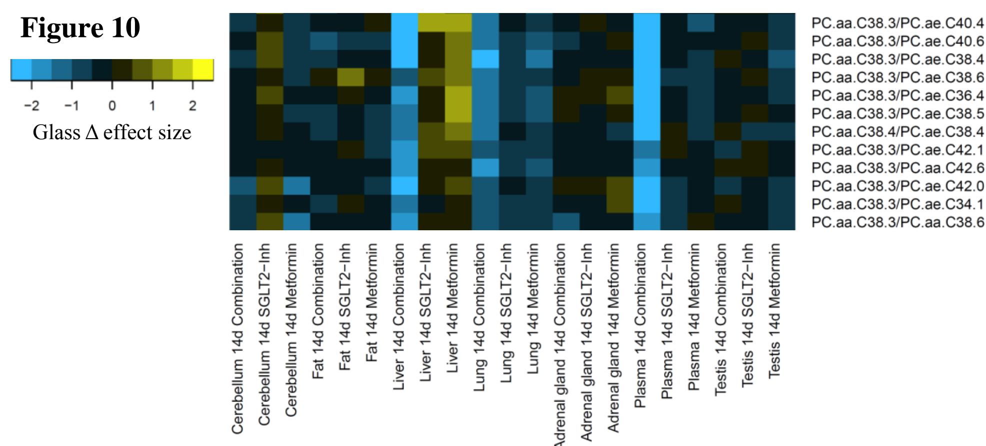


Figure 10: Drug induced differences in phosphatidylcholine and lysophosphatidylcholine levels during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

3.3.1.4. Drug induced changes in sphingomyelin related profiles

Changes in sphingomyelin (SM) homeostasis were related to T2D and cardiovascular disease [83], [151]. Therefore, the influence of the different antidiabetic treatments on SM metabolism was evaluated.

Analyzing the sphingomyelin related results after 4 h acute treatment no single SM concentration changed significantly in any of the observed groups. Most of the significant altered metabolite ratios were found for PC/SM and lysoPC/SM ratios (Appendix 6.1.1).

Table 8: Alterations in the ratios between total SM and non-hydroxy SM to hydroxy SM in plasma of sub-chronic treated mice

	plasma			
	Total SM/Total SM(OH)		Total SM nonOH/Total SM(OH)	
	q-value	effect size	q-value	effect size
Metformin	0.0317	-1.73	0.0315	-1.73
SGLT2I	0.9581	-0.35	0.9581	-0.35
Combination	0.0038	-1.75	0.0038	-1.75

The sub-chronic treatment had only minor significant effects on the here observed organs. The most sever changes were observed in plasma during the metformin and combination treatment. In general, the total amounts of SM were not changed significantly in organs or plasma of one of the groups. In lung of combination treated mice, concentration of SM C16:1 was found to be significantly decreased independently from plasma. One SM species SM(OH) C22:1 was decreased in plasma of combination treated mice. The SGLT2I groups exhibited no alterations in their metabolite profiles (Figure 11).

Interestingly, the ratio between hydroxy and total sphingomyelin, as well as the ratio between non-hydroxy and hydroxy sphingomyelin was significantly altered in plasma of sub-chronic metformin and combination treated mice (Table 8). These alterations were observed only in plasma of the sub-chronic group but not in any of the organs.

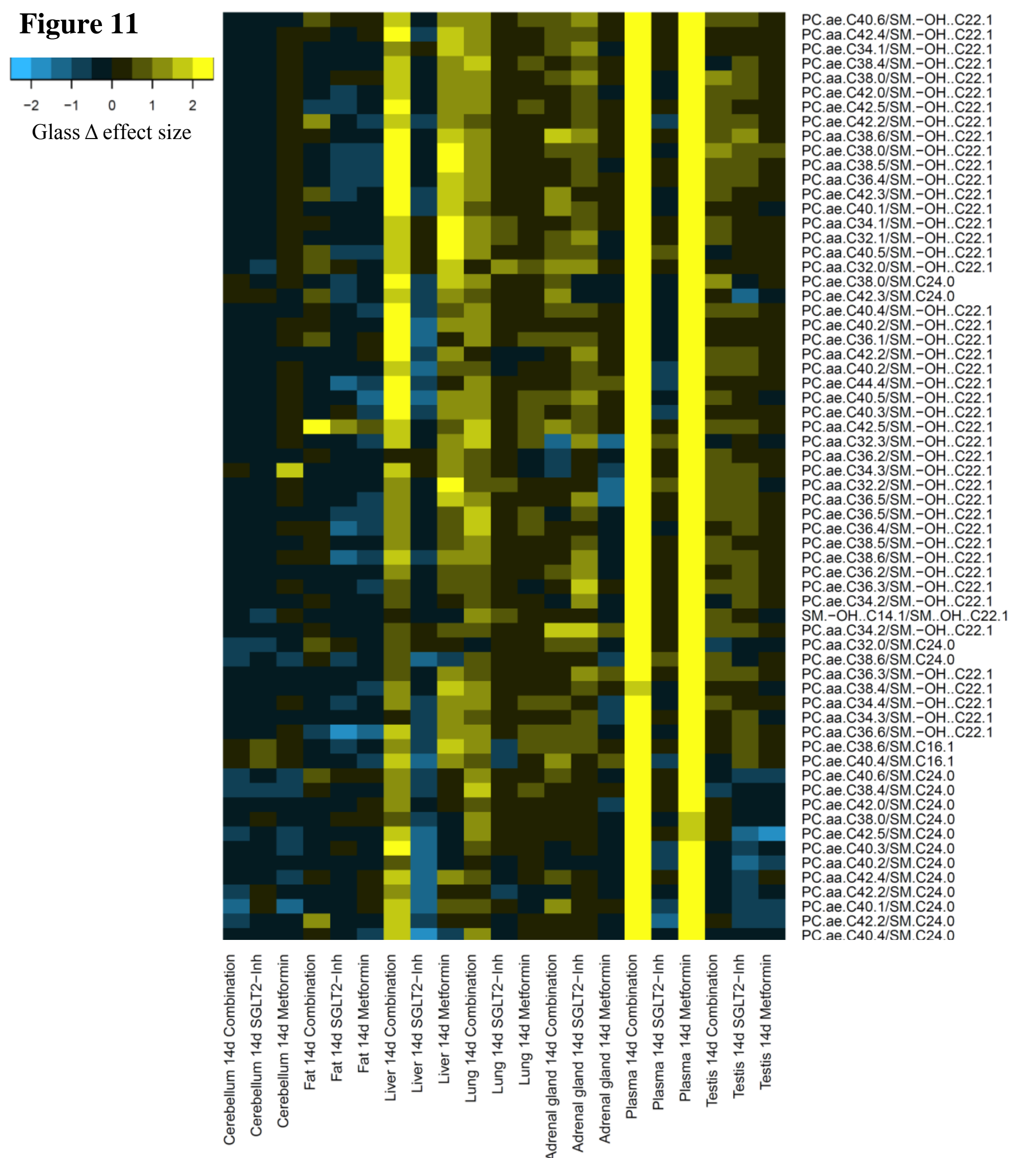
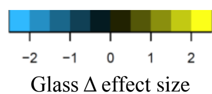


Figure 11



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PC.ae.C44.5/SM.C24.0
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PC.ae.C36.5/SM.−OH..C22.2

Cerebellum 14d Combination
Cerebellum 14d SGLT2−Inh
Cerebellum 14d Metformin
Fat 14d Combination
Fat 14d SGLT2−Inh
Fat 14d Metformin
Liver 14d Combination
Liver 14d SGLT2−Inh
Liver 14d Metformin
Lung 14d Combination
Lung 14d SGLT2−Inh
Lung 14d Metformin
Adrenal gland 14d Combination
Adrenal gland 14d SGLT2−Inh
Adrenal gland 14d Metformin
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Testis 14d Metformin

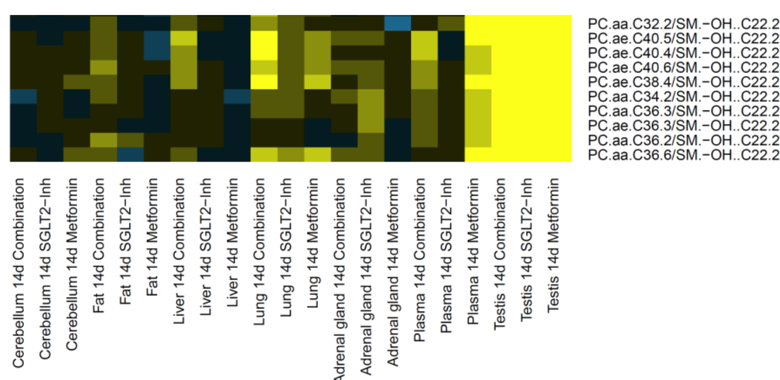

Figure 11: Drug induced differences in sphingomyelin levels during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

3.3.1.5. Drug induced changes in miscellaneous metabolite profiles

Not all metabolite ratios belong to one of the main classes. These metabolite pairs will be presented here.

During the acute treatment the most affected sites were plasma, testis, liver and adrenal gland. Strong changes were observed for SGLT2I and in part for combination treatment and some changes during acute metformin intervention (Figure 12).

The metabolite class of polyamines was one of the most affected ones. This group of metabolites is of interest, as perturbations in the polyamine pathway are associated with various pathological conditions, including diabetes [152]. Strongest effects were observed for AA/Putrescine ratios in testis, adrenal gland and liver in the SGLT2I, and in part in the combination treatment groups. In all three tissues, the ratios of AA/Putrescine were found to be elevated and putrescine concentrations were slightly decreased. In contrast to this, plasma ratios for this two treatment groups were decreased, and putrescine concentration elevated. Spermine and spermidine concentrations were increased during the SGLT2I and combination treatment with strongest effects in plasma and liver, and much weaker in testis and adrenal gland (Figure 12).

As described in chapter 3.3.1.2 Cit is a product of arginine metabolism. The same is true for putrescine. Interestingly, the Cit/Putrescine ratio was significantly elevated in adrenal gland. An equally strong, but not significant increase was observed in liver, indicating a relation between both metabolites. In contrast to the tissue specific observations, plasma Cit/Putrescine ratio were strongly but not significant decreased (Figure 12). This indicated a competition of different metabolic pathways for Arg (Figure 19 and Figure 20).

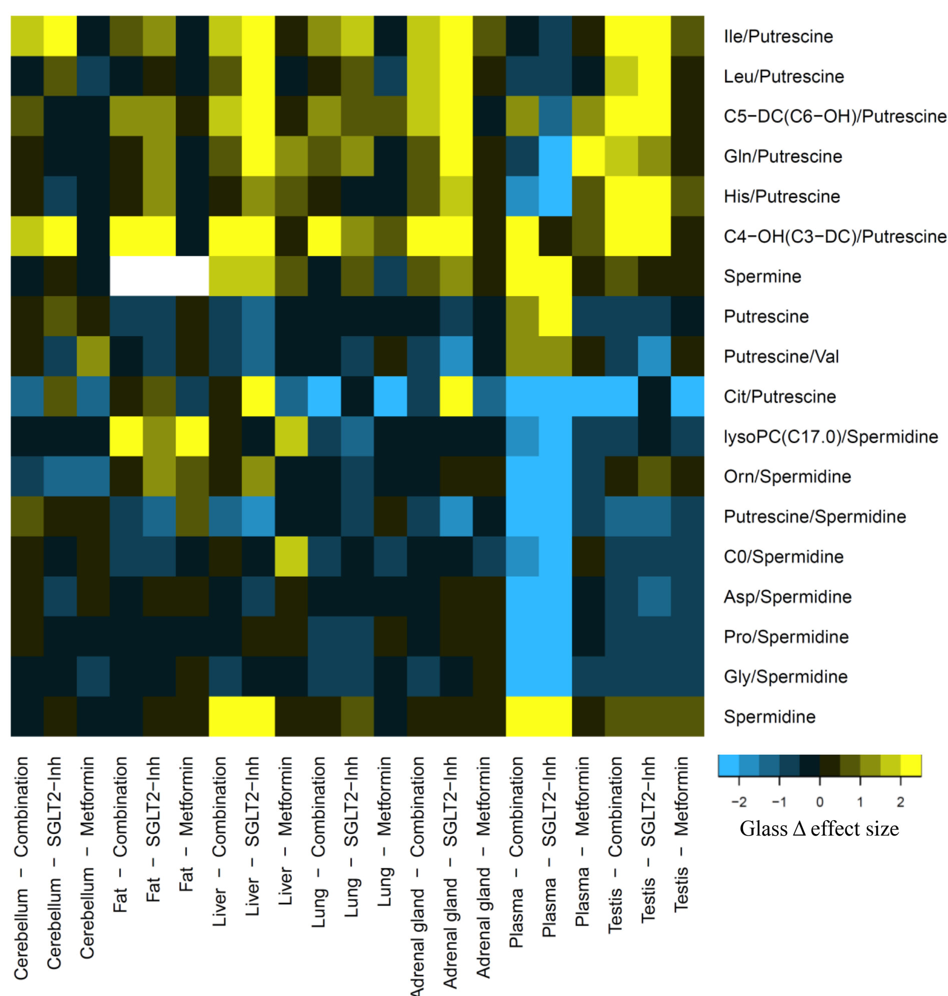

Figure 12: Acute changes in polyamine homeostasis: For acute treatment, 8 week old db/db mice were treated with a single dose of drug or vehicle. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the treatment groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

The sub-chronic antidiabetic intervention had significant impact only on testis (Appendix 6.1.2). For the PC/Putrescine ratios, a tissue specific downregulation can be observed in the testis sample from combination treated and in part from metformin treated mice. The plasma profile exhibit a clear but opposite regulation pattern for the PC/Putrescine ratios, indicating a real tissue specific reaction which was not influenced by systemic alterations of the metabolite concentration. Additionally, Putrescine/SM ratios were increased in testis and adrenal gland of combination treated mice (Figure 13). Together with the decrease in PC/Putrecine ratios this indicates an increase in putrescine levels in testis.

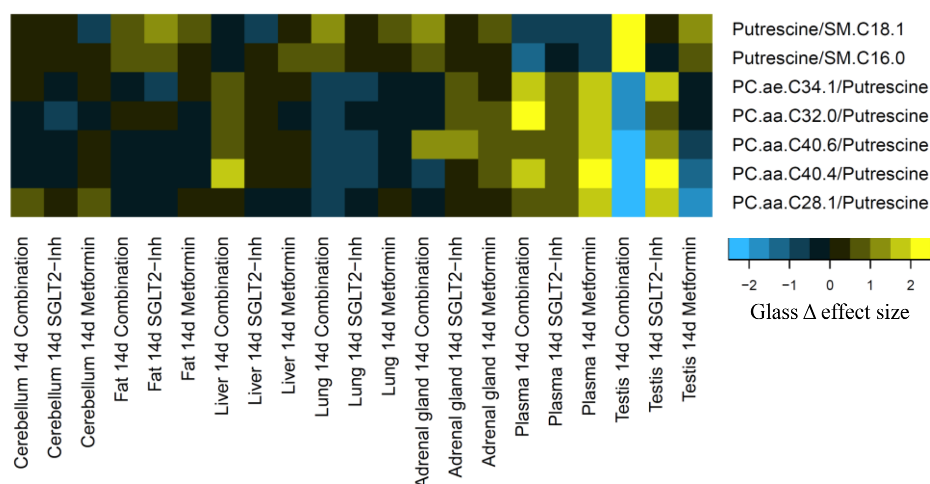

Figure 13: Sub-chronic changes in polyamine homeostasis. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

During the sub-chronic drug intervention, methionine sulfoxide (MetSO) homeostasis was affected. MetSO/PC ratios were significantly changed only in cerebellum and adrenal gland (Appendix 6.1.2). However, MetSO/PC ratios appear with strong effect sizes also in testis within the metformin and combination treated groups. The metformin treatment is here clearly the cause of action; because it led to the strongest alterations and the effect of the combination treatment was much weaker (Figure 14, Appendix 6.1.2). MetSO was not detectable in fat and liver samples.

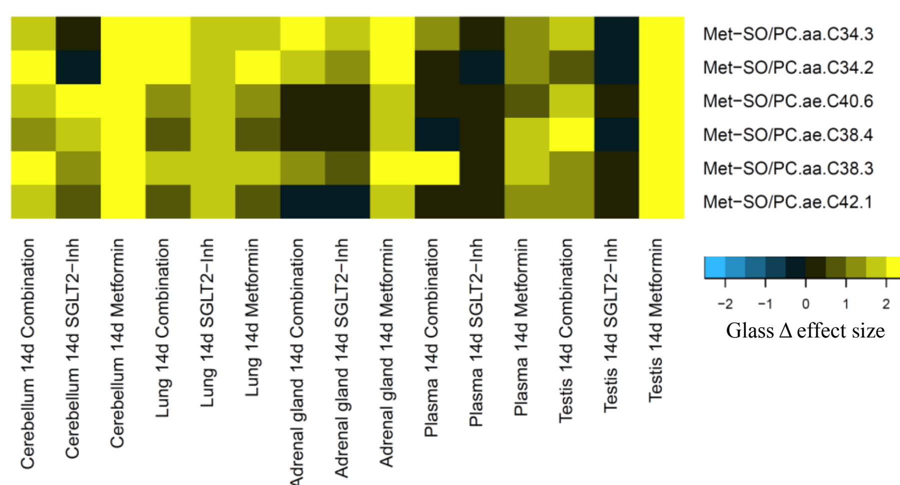

Figure 14: Alterations in MetSO homeostasis during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

Taking a closer look on the sub-chronic Histamine/PC ratios, the strongest alteration was found during the combination treatment in cerebellum samples. In the other tissues and treatments, no significant alteration was observed. The plasma profile of combination treatment only slightly reflects this alteration, indicating a cerebellum specific decrease of histamine concentrations (Figure 15).

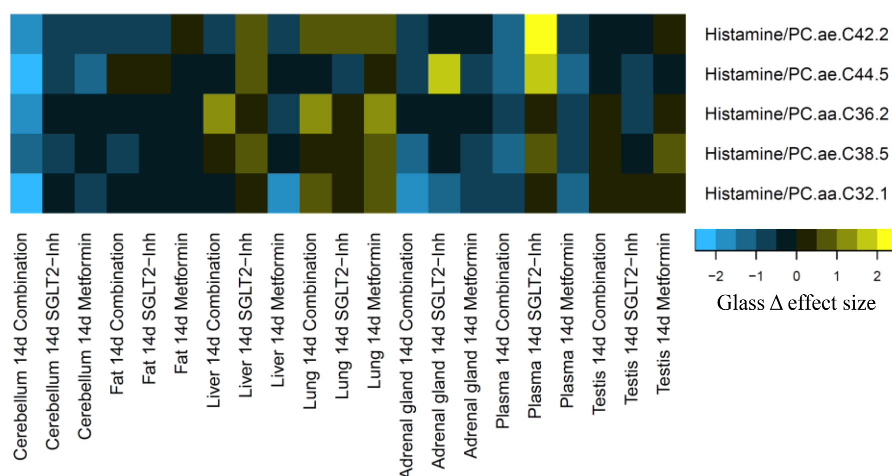

Figure 15: Alterations in Histamine homeostasis during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

The dipeptid carnosine and the Carnosine/Trp ratio were found to be significantly elevated in the cerebellum of sub-chronic combination treated mice (Appendix 6.1.2). Carnosine can act as antioxidant and has antiglycating properties. Therefore it is of interest for diabetes research. Additionally, carnosine homeostasis is linked to histamine, as it has been reported that carnosine can serve as reservoir for histamine precursor [153]. In [153] it was shown that carnosine and histamine were inversely regulated during stress. The same was the case for the here observed histamine and carnosine levels. The strong cerebral response in carnitine homeostasis to the combination treatment was tissue specific and not observed in any other organ or in plasma (Figure 16). Only in testis of the metformin treated group and in part in plasma of the combination treated group, a not significant elevation of carnosine was observed (Figure 16). Carnosine was not detected in fat, liver or adrenal glands.

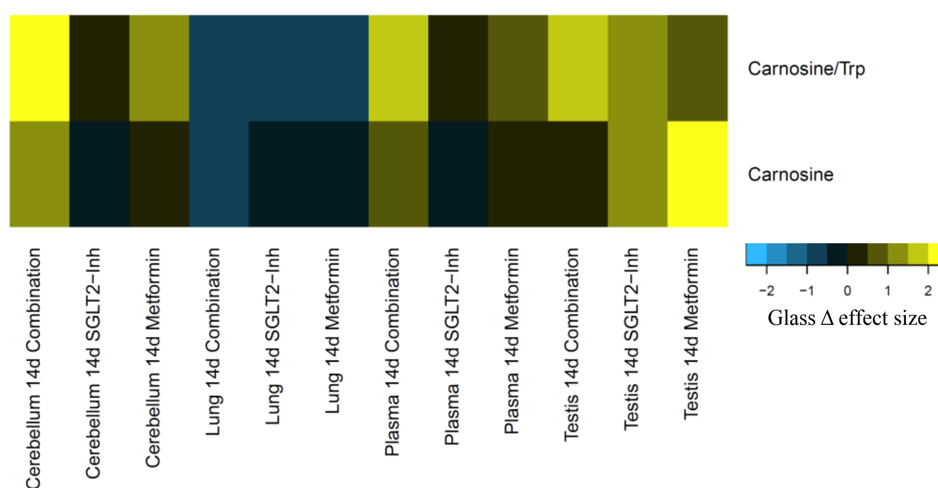

Figure 16: Alterations in Carnosine homeostasis during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

As described in paragraph 3.3.1.1, the degradation of Lys was altered. One of the intermediate metabolites in the Lys degradation process is alpha amino adipic acid (alphaAAA) [154]. The levels of alphaAAA were found to be significantly enhanced only in plasma of the sub-chronic combination treated mice but in none of the organs tested in this study (Appendix 6.1.2). This alteration was accomplished by an elevation of alphaAAA/AC ratios, and by an increase of Lys and alphaAAA/Trp levels (Figure 17). The pattern observed in Figure 17 indicated that the same effect occurred in plasma of metformin treated mice. This indicates that metformin treatment was the main cause of this effect. However, the combination treatment results in a stronger and significant alteration of alphaAAA homeostasis. This demonstrated a additional effect of the combination treatment above the single drug intervention.

As shown in Figure 17 a similar but not significant response of Lys, Trp, and alphaAAA were observed in testis.

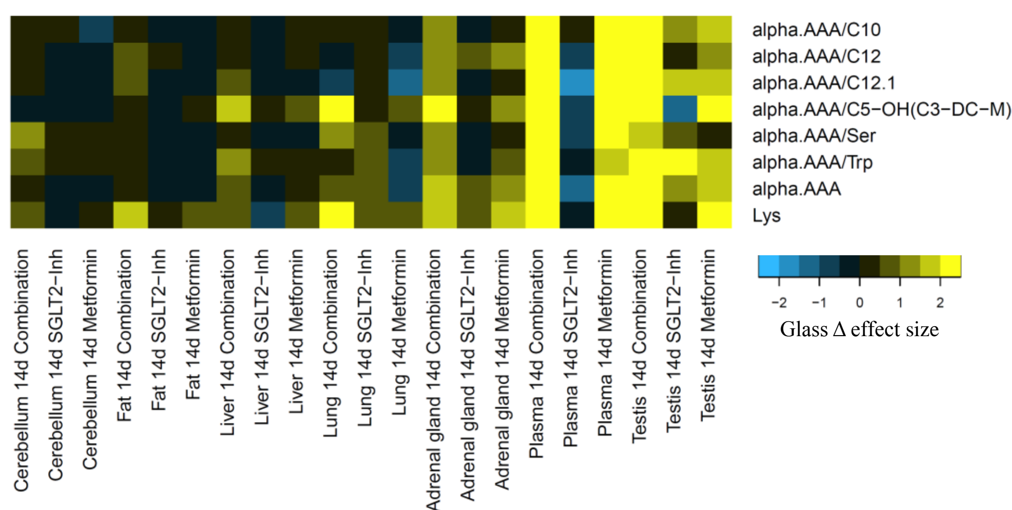

Figure 17: Alterations in alphaAAA homeostasis during sub-chronic treatment. For the sub-chronic treatment, 8 week old db/db mice were drug or vehicle treated for 14 days. For all presented groups, the GlassΔ effect size compared to the vehicle treated db/db mice group were shown. Only GlassΔ effect sizes of metabolites and metabolite ratios which showed significant alterations in at least one of the groups were presented. Effect sizes exceeding 2.5 were set to 2.5 or -2.5, respectively.

3.3.2. Characteristic metabolic pattern specific during sub-chronic combination treatment

The potential of metabolomics to identify characteristic metabolic signatures [155] was used to investigate the metabolic signature which discriminates the long-term blood glucose decrease during the sub-chronic combination treatment from the single drug treatments. Therefore, the metabolomics patterns in the combination treated animals were compared to the single drug treatments. Different metabolite patterns occurred specifically only during the sub-chronic combination treatment. They were either tissue specific or appear simultaneously in multiple sites of the body.

The most prominent pattern appearing in most tissues and plasma was the change in phosphatidylcholine- and lysophosphatidylcholine ratios indicating an increase in chain length and unsaturation of fatty acids as described in chapter 3.3.1.3. The changes were also observed in the metformin treated groups but more pronounced during the combination treatment.

The next observed massive pattern is the shift from PCaa to PCae which was observed mainly in lung, plasma and liver as described in chapter 3.3.1.3.

Some single metabolites only changed significantly during the combination treatment. They are listed together with their site of action in Table 9. The global effected metabolites were found within the lysoPCs. lysoPC(18:1) and lysoPC(18:2) were clearly enhanced in all tissues and plasma, followed by

lysoPC(20:4) and lysoPC(16:0), which were enhanced in lung and cerebellum.

Cerebellum exhibit a specific upregulation of 4 PC species (Table 9) and lysoPC(16:1), for which no effect were observed in plasma (Figure 18). Additionally, free carnitine (C0) and Carnosine were found to be tissue specific up-regulated in the cerebellum.

Table 9: Significant altered metabolites during combination treatment
Elevation of metabolite levels in the sub-chronic group compared to the vehicle treated group were indicated by ↑, a reduction by ↓, respectively.

metabolite		site of action	metabolite		site of action
C0	↑	cerebellum	**alpha.AAA**	↑	plasma
Carnosine	↑	cerebellum	**C10**	↓	plasma
lysoPC.a.C16.1	↑	cerebellum	**C18**	↓	plasma
PC.aa.C36.5	↑	cerebellum	**C4.1**	↓	plasma
PC.aa.C36.6	↑	cerebellum	**Cit**	↑	plasma
PC.aa.C38.5	↑	cerebellum	**PC.aa.C32.0**	↑	plasma
PC.ae.C38.0	↑	cerebellum	**PC.aa.C36.1**	↑	plasma
PC.ae.C44.3	↑	cerebellum	**PC.aa.C40.2**	↑	plasma
PC.ae.C44.5	↑	cerebellum	**PC.aa.C42.2**	↑	plasma
C5.1	↑	lung	**PC.ae.C34.0**	↑	plasma
Met	↑	lung	**PC.ae.C34.2**	↑	plasma
lysoPC.a.C17.0	↑	lung	**PC.ae.C34.3**	↑	plasma
PC.aa.C34.2	↓	lung	**PC.ae.C36.0**	↑	plasma
PC.aa.C34.3	↓	lung	**PC.ae.C36.1**	↑	plasma
PC.aa.C36.3	↓	lung	**PC.ae.C36.2**	↑	plasma
SM.C16.1	↓	lung	**PC.ae.C36.4**	↑	plasma
			PC.ae.C38.1	↑	plasma
lysoPC.a.C16.0	↑	lung/cerebellum	**PC.ae.C38.2**	↑	plasma
lysoPC.a.C20.4	↑	lung/cerebellum	**PC.ae.C38.6**	↑	plasma
lysoPC.a.C18.1	↑	lung/cerebellum/plasma	**PC.ae.C40.2**	↑	plasma
lysoPC.a.C18.2	↑	lung/cerebellum/plasma/testis	**PC.ae.C40.4**	↑	plasma
PC.ae.C42.3	↑	cerebellum/plasma	**PC.ae.C42.5**	↑	plasma
			PC.ae.C44.6	↑	plasma

The specific changes observed in lung were in part quite unique from all other observed sites of action. The combination treatment led to an increase in odd chain fatty acids, reflected by increased amount of C5:1 and lysoPC(17:0). Even most long-chain unsaturated PC levels were found to be elevated in plasma, 3 distinct PCaa concentrations were decreased in the lung. Additionally, SM C16:1 concentration was decreased and Met concentration increased during the combination treatment.

The single PC significantly changed in plasma reflected the shift to more PCae, and FAs with longer chain-length with higher degree of unsaturation. One exception was the increased PCaa32:0 level. As shown in chapter 3.3.1.2, Cit levels were enhanced specifically in plasma. Furthermore, 3 acylcarnitine concentrations were decreased. AlphaAAA was significantly elevated in plasma (Table 9), but showed an intense but not significant increase in testis (Figure 18).

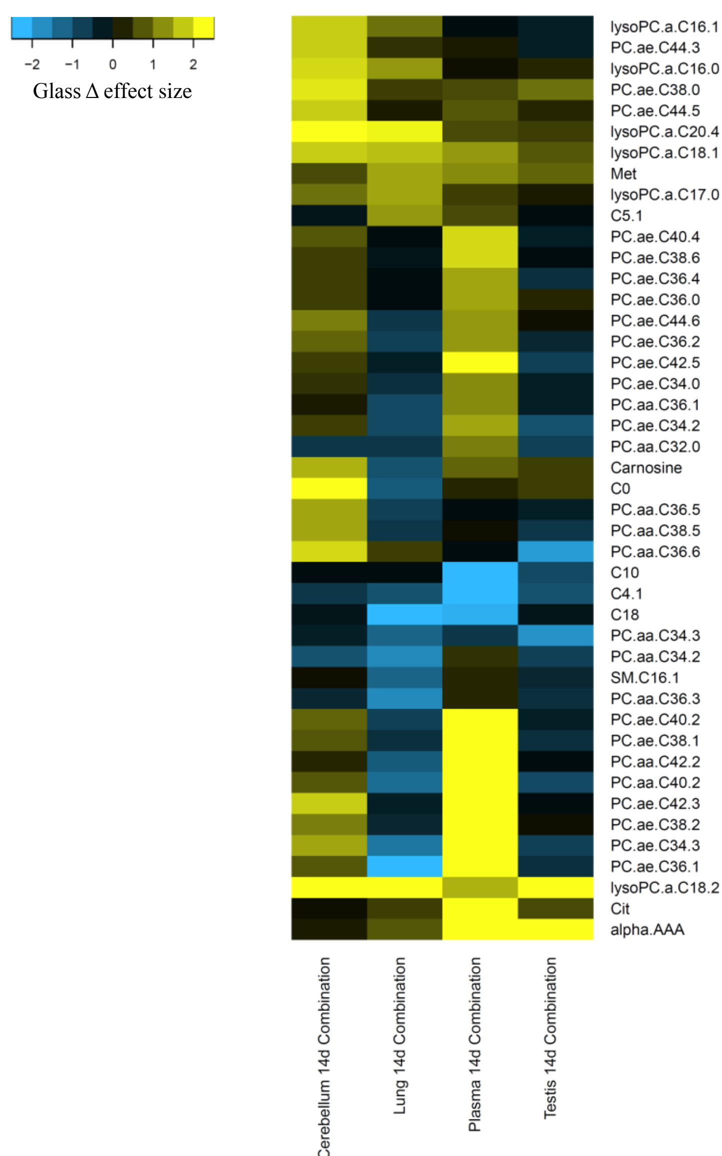

Figure 18: Significant alteration during combination treatment. Combination therapy specific changes for single metabolites during the sub-chronic treatment in db/db mice. Metabolites which showed significant alterations in at least one of the tissues were presented. Positive effect size means elevation of concentration in the treated group compared to the db/db vehicle treatment.

4. Discussion

The approach to investigate drug induced changes in metabolite homeostasis and antidiabetic drug pharmacokinetics by mass spectrometry based assays was in the overall very successful. However, the use of a diabetic mouse model can only in part reflect the human metabolomics response to antidiabetic intervention. In fact, while the human metabolome profiling is observational, the mouse model can be challenged. Therefore, the mouse model allows the tracing of metabolite homeostasis and drug pharmacokinetic down to tissue specific alterations in response to standard and novel antidiabetic drug treatments.

4.1. Metformin quantification

4.1.1. Setting up the LC-MS/MS quantification

The use of LC-MS/MS technique with electrospray ionization allows the highly sensitive detection and quantification of metformin in mouse tissue and plasma. The quantification of metformin in tissue from diabetic mice was done before with a radiolabeled ^{14}C assay using similar concentration and the same time points [71]. The results from the ^{14}C assay were within the same concentration ranges as the here presented ones, indicating the reliability of the method.

The assay already showed its applicability for the measurement of metformin in tiny amounts of tissue, as it was successfully applied to mouse retina samples [156]. Therefore the method offers a highly sensitive, selective, and reproducible quantification of metformin from mouse tissues and plasma together with an easy sample preparation. One point during assay development was its applicability to samples prepared for metabolomics measurements. The assay is clearly adaptable to a wide range of samples prepared for general metabolomics measurements as the here used extraction procedures in methanol or ethanol mixed with aqueous solution are commonly used for unbiased metabolite extraction [78], [79].

4.1.2. Pharmacokinetic interaction of metformin and SGLT2I

SGLT2Is offer a potential treatment for T2D in combination with existing therapies. The inhibition of the kidney SGLT2 transporter leads to decreased reabsorption of glucose in the kidney, but also enhances polyuria. This, in fact can alter the pharmacokinetic of other admitted drugs. The experiments done here were not adequate to test the complete pharmacokinetic (like area under the curve) of metformin as only two time points (4 hour, 18 hours) were investigated. However, the co-administration of the SGLT2I led to significantly lower metformin concentrations in plasma, liver, as well as in the main target kidney, at both time points. As metformin plasma pharmacokinetics are well described [157], it is clear that the here used SGLT2I lower the peak plasma concentrations (after 4 hours) as well as accelerates the concentration drop during the elimination phase. Therefore, it is possible that the decrease in metformin results in a biological or clinical relevant alteration of metformin action.

The here presented decrease of metformin concentrations, was contradictory to published results. [158] showed that the co-administration of metformin and the SGLT2I remogliflozin etabonate had no effect on the pharmacokinetic of metformin in human diabetic patients.

SGLT2Is are known to inhibit the organic cation transporter (OCT1) [159] which is responsible for the uptake of metformin into cells and tissues. As lung, cerebellum, and 4h treated testis showed no alteration in metformin uptake, it is unlikely that OCT transporters are affected. More likely is that plasma concentrations of metformin were altered by enhanced excretion. The increased excretion will immediately effect the concentrations in plasma, liver and kidney as observed in the results.

The here presented findings indicate an altered metformin pharmacokinetic. Therefore, human studies are needed to investigate the influence of the SGLT2I in clinical use and may adapt the metformin doses to ensure stable blood glucose levels during long-term drug intervention.

4.2. Pharmacometabolomics

Purpose of this part of the thesis is to get insight into metabolic changes in a diabetes mouse model during antidiabetic intervention, focusing on changes in peripheral organs and systemic alteration reflected in blood plasma. This is studied by a targeted metabolomics approach, focusing on the pattern of small biological molecules in response to pharmaceutical intervention. This concept is coined as pharmacometabolomics [160]. A major potential in the application of metabolomics to the investigation of drug action involves the tracing from the biochemical pathway back to particular

enzyme. The analysis of metabolomic signatures during drug treatments is already used to identify biochemical drug targets [161]–[165]. The results from such studies can confirm what is already known about the effect of the drug. However, their power lies in the possibility to identify new pathways that are affected and which possible future targets for new drugs. Additionally, in complex disease as T2D or the metabolic syndrome, the action of a drug may not be restricted to the primary target but can be also beneficial to related diseases, enlarging the therapeutic spectrum of a drug.

4.3. Specificity of observed metabolomic pattern

Metabolite tissues homeostasis is no stand-alone mechanism, rather exists a dynamic equilibrium with the surrounding biofluids. Tissue specific metabolomics changes may be only in part reflected in the plasma or other biofluids. A specific tissue may not exchange a metabolite with the plasma or the concentration of the exchanged metabolite is too low to cause a detectable change in plasma levels. On the other side, the tissue specific metabolite pattern can be influenced by metabolite uptake from the plasma.

Organ specific differences in metabolite concentration and different tissue specific extraction properties can cause substantial differences in the detected metabolite pattern. Some of the here observed differences in metabolic pattern between the tissues may be caused by a lack of measureable metabolite concentration in one of the tissues. Furthermore, tissue specific technical reproducibility (CV) for the metabolites, alter the overall variance of the measurement, resulting in different outcomes of significance tests.
Cerebellum, testis, adrenal glands are organs containing different distinct structures. Here the whole response to treatment or diabetes status is observed. But the observed reaction perhaps occurred due to changes only in one distinct part or one species of cells within the whole organ.
Comparing the tissue specific and systemic (plasma) alteration in the metabolite profiles, the vast majority of effects were detectable in plasma. Plasma clearly not reflects all tissue specific metabolic patterns which showed up, but not all tissues were investigated here and they may have an impact on the systemic alterations.

What should be mentioned here is the fact that the samples were withdrawn without perfusion. The perfusion of organs with a buffer solution is generally used to completely remove the blood. However, there are some drawbacks while performing perfusion, especially to metabolomics studies. The prolonged time at physiological conditions prevent a fast quenching of the metabolism. Additionally the perfusion can alter the metabolome of the organ of interest. Therefore, only complete blood removal from the whole body via the punctuated vena cava was performed here. This procedure may

be leads to some remaining blood in single tissues. The blood metabolome can be interfering with the investigated metabolome of the organ, causing a dilution effect or leading to the detection of non-organ specific metabolites or metabolite concentrations. As [166] had shown for selected liver metabolites, the influence to most metabolites is negligible. Additionally, the metabolite signatures found in the here observed organs do not generally overlap with the plasma signatures, which indicates that here is no or less influence between plasma and organ metabolome. However plasma metabolome is not the blood metabolome and the concentrations of single metabolites may different between plasma and blood [167] and not covered by the plasma versus organ comparison.

4.4. Glucose lowering effects of the antidiabetic treatments

The primary aim of the treatment of diabetes is to normalize blood glucose levels which are enhanced in diabetes by unrestrained endogenous glucose production and peripheral insulin resistance. Despite the fact that T2D is a multifactorial disease, different treatment strategies are used to reach this aim. If one therapy fails to reach glycemic control, the combination of antidiabetic drugs can be employed to achieve normal glucoses levels. The application of SGLT2Is presents a novel way to immediately decrease blood glucose levels by the fast excretion of glucose via the urine. In combination with metformin it may provide an efficient glycemic control.

During the acute treatment phase normal plasma hexose levels were faster achieved by SGLT2I and combination treatment than by metformin alone. Here SGLT2I is responsible for the majority of the glucose lowering effect during the acute treatment in combination therapy.
The sub-chronic SGLT2I treatment had no significant effect 18 hours after the last administration. The same was true for metformin, even if there was a modest decreases in plasma hexose concentrations, which goes in accordance with the results from [168]. SGLT2I and metformin decreased plasma hexose levels in the acute treatment, but failed in long-lasting effects in sub-chronic treatment. Only sub-chronic combination treatment resulted in a decrease of plasma hexose levels which were comparable to the wildtype levels and to those observed during acute SGLT2I and combination treatment. As concluded in [168], this effect was supported by the metformin induced decrease in endogenous glucose production during the combination treatment compared to the single SGLT2I therapy. In conclusion, only the combinatory action of metformin together with SGLT2I improved glycemic control by lowering blood glucose levels in a long-lasting effective manner.

It should be mentioned, that the here measured metabolite parameter was the sum of all hexoses and not only the glucose level. However, the observed effects of the antidiabetic treatment on plasma

hexose levels were in accordance with published results for plasma glucose levels from this experiment [168]. Therefore, the measured plasma hexose level equals plasma glucose concentration.

Except liver, the hexose levels in tissues closely followed the plasma levels. Therefore, peripheral tissues were not involved in the main glucose production (liver) and uptake (muscle) process, but mainly rely on plasma glucose. They did not produce large amounts of glucose nor had an increased uptake of glucose beyond the level of blood glucose and their hexose level reflected the plasma level.

Compared to plasma and the other observed organs, the hexose levels in liver showed much weaker increase from the non-diabetic wildtype to the diabetic animals, indicating that glucose utilization and accumulation in the liver were only weakly effected by the diabetic phenotype. Additionally liver hexose levels showed only modest response to the different treatments. However, they were found enhanced during acute metformin treatment. Only few studies were performed on acute metformin treatment, most of them were clamp studies with insulin or pyruvate infusion and none of these evaluated glucose or hexose levels directly in the liver. For cancer cells, data from [169] indicate a slightly increased uptake of glucose shortly after the treatment with metformin, followed by a decreased uptake over time. Together with the data from [170] which clearly indicate an increase of glucose utilization, but also increased glycogenolysis in the acute response to metformin this may explain the enhancement of liver glucose levels during the first phase of the acute treatment.
Like in plasma and the other organs, only sub-chronic combination treatment resulted in a markedly decrease of hepatic hexose levels which were comparable to the wildtype level. Again, glucose lowering in all investigated peripheral organs and liver were only achieved by combination treatment.

In conclusion, the best systemic and organ specific glycemic control was achieved by the combination treatment (Figure 3). Improved blood glucose (Figure 2) and HbA1c [168] level indicating a profound change in glucose utilization and energy metabolism in a long-lasting manner.

4.5. Acute and sub-chronic pattern of antidiabetic drug intervention

In this part of the thesis the effects of the individual and combined treatment with antidiabetic drugs on metabolite alterations have been studied. The drugs, tested in this study, act via different sites and modes of action [23], [39], [171], [172]. Metformin was described to act mainly on the gluconeogenesis in the liver, but its cellular mode of action is the inhibition of complex I from the respiratory chain [23]. Consequently, all organs are potential targets of metformin action. The SGLT2I (AVE2268) acts very selective on one cellular transport system (SGLT2) which is expressed mainly in kidney and gut [40]. However, the SGLT2I induced fast and massive depletion of blood sugar levels

[46] which may also result in multiple systemic and organ specific metabolic responses. A combination of treatment strategies is widely used in diabetic treatment [173]–[178]. The combined administration of metformin and the SGLT2I may cause combination effects, which goes beyond the above observed glycemic control (paragraph 4.4). These effects can result in additional, stronger, or quite different alterations in biochemical homeostasis than the single drug treatment dose [168], [179].

The acute treatment resulted in strong alterations, mainly in AA and AC homeostasis, caused by SGLT2I or combination treatment. Metformin treatment had only a minor impact to the here observed metabolites. This indicates that the fast action of the SGLT2I was mainly responsible for the alterations observed during the combination treatment.
Analyzing the effects of the sub-chronic intervention, metformin and combination therapy were the causes for the metabolomic alteration. The sub-chronic intervention with the SGLT2I for 14 days did not affect the metabolite profiles in a significant way, neither tissue specific nor on the systemic level. The SGLT2I treatment did not target the pathological mechanisms of diabetes or the metabolic syndrome directly. The primary effect of the SGLT2I is the regulation of blood glucose levels by increased renal glucose excretion. This mimics a carbohydrate restriction, and caused a massive energy deficit. However the observed alterations in metabolite homeostasis remain on a not significant level.
Combining the SGLT2I with metformin resulted in similar metabolite pattern as observed under single metformin treatment. However, some combination treatment specific alteration occurred, indicating that the SGLT2I intervention had an significant effect in the combination therapy.

The continuing application of antidiabetic drugs can lead to adaptation of the body to the pharmacological intervention and therefore to an impaired action of the drug. On the other hand, a successful normalization of glucose levels may result in an adaptation to the new energy status and in a normalized global metabolite homeostasis. Impaired drug actions after long-term administration are known for metformin. For the SGLT2Is no serious adverse or adaption effects are described. However, the long-term treatment may lead to a higher risk for infections of genitourinary tract [180] which in fact can cause adverse systemic reactions.

In the present thesis no definite adverse effects on metabolite homeostasis were observed. Moreover, metformin and still stronger the combined treatment of SGLT2I and metformin improve metabolic patterns in plasma and different organs in response to sub-chronic treatment in db/db mice.

4.6. Methionine sulfoxide homeostasis

The sulfur side of methionine can easily be oxidized, resulting in the formation of methionine sulfoxide (MetSO). Thus, the formation of MetSO can give a hind to the intercellular oxidation status [181], [182]. The oxidation of Met can be enzymatically reversed. This cyclic oxidation of Met serves as important antioxidant mechanism and provides important mechanism for regulation of enzyme function [183]. Therefore, the Met/MetSO ratio represent a stable endpoint for the investigation of oxidative stress [184] were an increase of Met/MetSO indicates lesser oxidative stress. Additionally, it was shown that chronic metformin treatment prevents memory impairment induced by Met, probably by reducing the oxidative stress in the brain [185]. Oxidative stress has been implicated as important in several pathophysiological events including cardiovascular disease and diabetes. It occurs often at specific sites and therefore associated with local abnormalities.
Methionine sulfoxide (MetSO) homeostasis was significantly affected by the 14 day metformin treatment in adrenal glands and cerebellum of db/db mice. In both tissues, changes in ratios containing MetSO were detected. The levels of MetSO alone, or the ratio of MetSO to Met did not reach the significant level. However, changes in MetSO level can be interesting since MetSO act as endogenous antioxidants.

The here occurred alterations in MetSO homeostasis were only indirect observed via the ratio of MetSO to several PCs containing long-chain PUFAs. PUFAs itself are susceptive to auto-oxidation processes which can degrade it in a non-enzymatic way. The alteration in the ratio of these ROS sensitive metabolites points to an alteration in the oxidative status of the cerebellum during the sub-chronic intervention.
Even as the here observed increase in MetSO is not detectable during acute treatment and found weak during the sub-chronic treatment, it may play an important role during long-term interventions. Taking a look on to the wildtype data, it becomes clear that the Met/MetSO cycle is important in diabetes. The metabolite levels in cerebellum were enhanced in the 10 week db/db mice, but missing the significance levels (Met: q-value 0.08, effect size 1.6, Met/MetSO ratio: q-value 0.059, effect size -1.7). The circulating levels of MetSO (q-value 0.0007, effect size 1.8) and MetSO/Met (q-value 0.0129, effect size 0.9) were clearly decreased in the 10 week old diabetic group. The marked decrease in Met/MetSO plasma levels indicate increased systemic oxidative stress in the diabetic animals. This goes in accordance with observations from insulin resistant mice [184]. However, the local response to diabetes in the brain is quite opposite to the systemic level.

The data from this thesis implicate a systemic alteration of MetSO homeostasis as well as alteration in cerebellum during the sub-chronic metformin treatment. It remains unclear which effect the metformin treatment did have to motoric and cognitive function and to the oxidative stress level in the brain [186].

4.7. Citrulline – Improvement to vascular function?

Increased levels of plasma Citrulline (Cit) were associated with renal failure [187] but also with improved nitric oxide synthesis [188]. Cit synthesis is facilitated by two enzymes: Ornithine Carbamoyl Transferase (liver, gut) and nitric oxide synthase (neuronal cells, macrophages, endothelial cells) (Figure 19). Degradation of Cit is catalyzed by argininosuccinate synthetase (all tissues) [189]. The supplementation of Cit has positive effects not only to blood pressure but can also ameliorate glucose and lipid levels and may improve insulin sensitivity in diabetes [190], [191]. Therefore, changes in Cit level during antidiabetic drug intervention may not only biomarkers for a successful treatment, but by itself beneficial to the metabolic syndrome and were therefore discussed in this thesis.

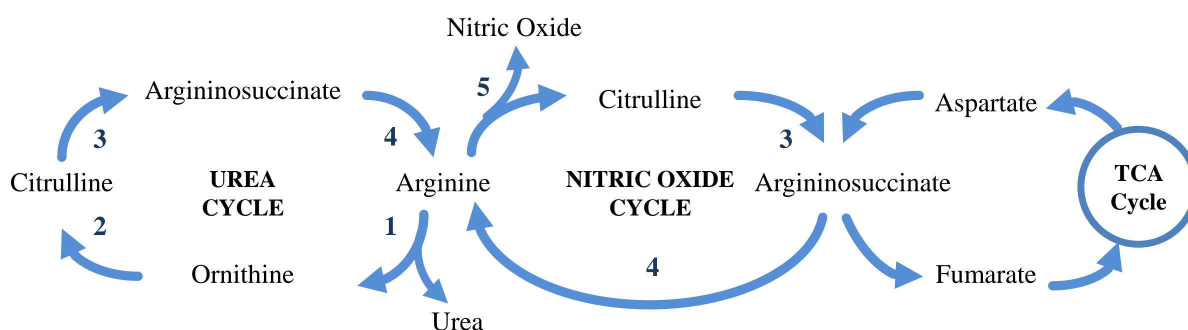

Figure 19: Citrulline turnover in urea and nitric oxide cycle.
In the urea cycle arginine is converted to ornithine by ArginaseI/II (1) and further to citrulline by Ornithine carbomyltransferase (2), which is converted to Argininosuccinate by Argininosuccinate synthase (3) and back to aginine by Arginosuccinate lyase (4). The first step in the nitric oxide cycle is the conversion of arginine to citrulline by Nitric oxide synthase (5). The conversion back to arginine uses the same steps as the urea cycle. The conversion of Aspartate to Argininosuccinate and further to Fumarate linking the Nitric oxide cycle to the Tricarboxylic acid cycle (TCA) [192].

Plasma Cit levels were found to be significantly higher after 14 day combination treatment compared to vehicle treated db/db group or wildtype mice. The main source of Cit in plasma is the vascular

endothelium, which produces Cit and nitric oxide by the conversion of arginine via the endothelia nitric oxide synthase (eNOS) (Figure 19). The observed enhancement in Cit level during the sub-chronic treatment is limited to plasma and was not observed in specific organs. This indicates a plasma or endothelium specific origin of the measured Cit in the sub-chronic treatment group. Cit is also produced in significant amounts by the urea cycle in the liver. However, animal and human data suggest that liver does not influence the circulating Cit levels neither by uptake nor by release [193], [194]. Therefore, liver can be excluded as source of the observed plasma Cit levels.

It is possible that the enhancement of Cit in plasma was induced by increased arginase activity instead of eNOS, this should result in enhanced Orn levels, which were not significantly altered during the sub-chronic treatment. Beside the stable Orn levels, it was shown that arginase activity is enhanced in aorta and liver of streptozotocin-induced diabetic mice, and normalized under normal glucose levels [195] like in this experiment.

As arginase compete with eNOS for arginine, they are in part responsible for a decreased eNOS activity. Additionally, metformin suppresses tumor necrosis factor (TNF) production in monocytes [196] and endothelia cells [197]. TNF itself contributes to endothelia dysfunction by upregulation of arginase activity and increased superoxide production. Inhibition of TNF clearly reduces arginase activity and improves vascular function [198]. Finally, the combination treatment resulted in a reduction of blood glucose levels by SGLT2I, together with reduced arginase activity by metformin. Both effect improve eNOS activity and therefore responsible for the here observed increased effect of the combination therapy.

The action of argininosuccinate synthetase can partially explain the observed elevated plasma Cit levels. They facilitate the degradation of Cit in the endothelium and were described to be up regulated in diabetes and restored to physiological levels by antidiabetic treatment [199].

Altogether, the observed sub-chronic enhancement of Cit together with published results indicates an upregulation of eNOS activity together with the normalization of the other enzymes involved in Cit homeostasis. Metformin itself is an activator of the endothelia nitric oxide synthase [200], [201] which causes higher nitric oxide levels. In the sub-chronic combination treatment, the decreased glucose levels can induces other beneficial effects enhancing eNOS activity, as decreased arginase activity. The improvement in nitric oxide levels lead to a normalization of blood pressure and improved vascular function [202]. Improved vascular function and elevated Cit levels can ameliorate glucose homeostasis [203] and induce insulin release [204]. Additionally, mutation in eNOS results not only in altered vascular reactivity but also modulate insulin sensitivity, insulin level and adiposity [205], making eNOS to potential drug targeted in cardiovascular disease and T2D.

Therefore, the observed elevated Cit levels in part explain the observed improvement of glucose homeostasis in the combination treatment above the single drug interventions. Eventually, metformin

and combination therapy do not only improve metabolic health, but additionally protects the cardiovascular system by reducing inflammation and oxidative stress.

Since the Cit levels are much higher in the plasma of combination treated animals compared to the wildtype animals, there is perhaps an overregulation of the Cit regulating pathways. If the proposed upregulation of eNOS is correct, adverse effects can occur if NO production is massively increased over a long time period. Permanent high NO levels may result in enhanced oxidative stress, due to the formation of peroxynitrite and therefore can counteract the beneficial effects in the vascular system.

The sub-chronic treatment with the SGLT2I can cause renal infection or dysfunction but only in rare cases and with mild or moderate intensity [172], [206], [207]. Therefore, it remains unlikely that the elevated levels of Cit are caused by renal impairment. The data of the single SGLT2I treatment implicate no change or a slight decrease of Cit in plasma (effect size -0.57, qvalue 0.93) indicating no SGLT2I specific effect. Therefore, a renal dysfunction induced by the SGLT2I action appears unlikely.

Cit levels were found to be decrease in testis, lung, and plasma of acute metformin and combination treated mice. These reverse reactions of the antidiabetic treatment may result from the acute action of the antidiabetic drug and indicate a disturbance of the biochemical homeostasis which finally result in an activation of healthy metabolism after continuous antidiabetic treatment in the sub-chronic group. On the other hand, the acute and sub-chronic groups receive their antidiabetic treatment different times before the end of the experiment. The alteration found may reflect the different concentrations of the antidiabetica at the time of organ withdrawal, or the different times the metabolism of the mice had to react to the treatment. The acute response to the SGLT2I intervention in liver increases Cit and Orn levels together and is therefore due to enhanced arginase or ornithine carbomyltransferase activity.

4.8. Polyamines

Polyamines are biogenic amines with two or more primary amino groups. Here the three polyamines putrescine, spermidine, and spermine were further investigated. The changes in polyamine homeostasis during the acute SGLT2I treatment indicates a decrease in putrescine levels in testis and adrenal glands, whereas plasma levels of putrescine and more stronger of spermidine and spermine increases. The acute combination therapy led only to weaker changes in polyamine homeostasis. Here metformin may counteract the action of the SGLT2I.

The main pathway for polyamine production is the conversion of Arg to putrescine and further to spermidine and spermine. Due to the fact that eNOS and polyamine synthesis need Arg as substrate,

the alteration in polyamine homeostasis can effect eNOS activity [208] (Figure 20). The here observed massive increase in plasma polyamine levels during the acute treatment add therefore another explanation to the above describes decrease in Cit level. Additionally, this assumption was supported by he observed decrease of plasma Cit/Putrescine ratios, which indicates a shift from Cit to putrescine metabolism.

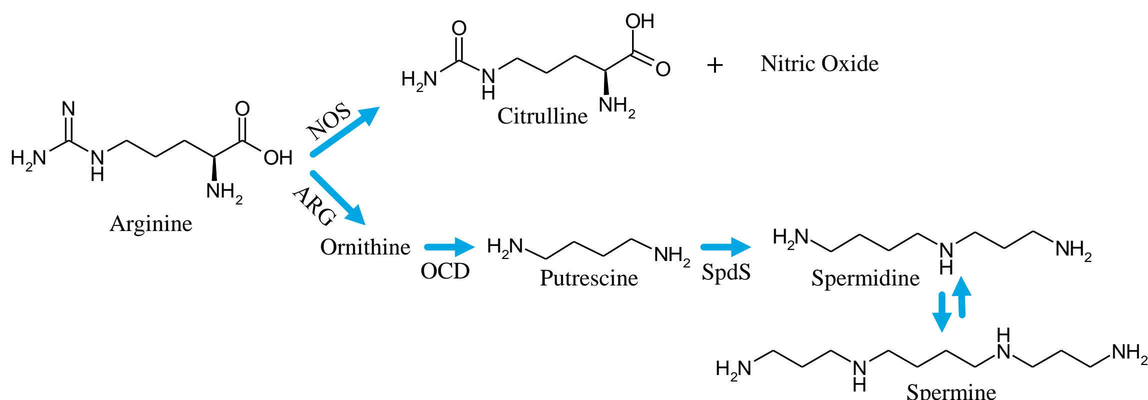

Figure 20: Arginine to polyamine metabolism.
The initial step in polyamine synthesis is the production of putrescine by ornithine decarboxylase (ODC). In the next step, spermidine synthase (SpdS) transfer an aminopropyl group to putrescine to form spermidine. Both, arginase (ARG) and nitric oxide synthase (NOS) compete for arginine as substrate. Therefore, increased polyamine synthesis can inhibit nitric oxide production.

During the sub-chronic treatment only an alteration of ratio from putrescine to PCs and SMs indicates an increase in putrescine levels in testis. As spermatogenesis is highly affected by diabetes, the normalization of glucose levels has a positive impact on sperm integrity. Beside the primary effect of antidiabetic treatment also elevated levels of polyamines protect the sperm cells from oxidative processes and provide structural integrity of DNA [209]. Therefore, even a slight elevation of polyamine together with normalized glucose levels can result in improvements of the reproductive system.

Interestingly, polyamine concentrations are high in mammalian brain [210], however the cerebellum specific polyamine homeostasis was not affected by the treatments. This indicates a brain specific regulation of polyamine homeostasis, independent from the systemic alterations.

Altogether, even if massive disturbances occur during the acute intervention, only weak and tissue specific long-lasting alterations were observed. Therefore, polyamine metabolism was only minor affected by the sub-chronic therapies.

4.9. Fatty acid and amino acid oxidation and turnover

Changes in acylcarnitines and amino acid homeostasis were related to obesity, diabetes, and antidiabetic therapy since several decades [211]. More recently, alteration in plasma acylcarnitine and BCAA levels comes again in the focus of current diabetes research [83], [98]. BCCA were linked to fatty acid synthesis and by this to insulin resistance and diabetes [98]. The biochemical pathways of fatty acids, acylcarnitines and amino acids merge in the energy producing reactions. If intervention, like antidiabetic treatment with SGLT2I causes alteration in energy homeostasis, both metabolite classes will show related regulation pattern.

The peroxisome proliferator activated receptor alpha (PPARα) is one of the main sensors of hepatic energy state. PPARα is a key regulator of fatty acid, acylcarnitine, glucose, and amino acid metabolism, and regulates mitochondrial as well as peroxisomal fatty acid oxidation [212] in various tissues [213]. Resent results demonstrate that metformin action can appear independently from AMPK, but depend on PPARα [38]. However, the metformin mediated activation of AMPK also results in a activation of PPARα and its downstream signaling [214].

Resent cohort studies identified biomarker profiles which distinguish metabolic well individuals from such with increased risk of developing metabolic syndrome or related diseases. The major discriminating factor between the groups comprises BCAAs and several acylcarnitines including C3 and C5 [215]. As C3 and C5 AC were generated during BCAA degradation both metabolite groups are closely related.

4.9.1. Acute action of metformin

During the acute metformin treatment the acylcarnitine and AA levels and ratios showed no alterations. Metformin acts via AMPK and PPARα signaling pathway. The acute treatment with a single dose seems to not alter these pathways in a sever way, as only slight changes in metabolite homeostasis were observed. From tissues only testis and lung from 4 hour metformin treated animals showed some punctual alteration at all. Changes in plasma are mainly restricted to modifications of three acylcarnitine levels (C3, C16.OH and C18.1OH). Only the continuing metformin treatment resulted in profound alteration of the biochemical pathway of peripheral organs. This is reflected in the results from tissues.

4.9.2. Does periodic energy restriction induced by SGLT2I improve metabolic health?

In contrast to acute metformin and combination treatment the acute SGLT2I treatment radically lowers the blood glucose levels and immediately saps the energy from the body. This causes a systemic change from glucose utilization to alternative energy resources. The same effect, even over several hours, occurs during fasting or caloric restriction. This is reflected in the here observed massive increase of long-chain acylcarnitines in the plasma and decrease in the liver, indicating a release of energy-rich acylcarnitines from the liver to plasma to overcome the glucose shortage in the body. This occurred together with a decrease of short chain AC and free carnitine in plasma. Depletion in free carnitine reflects the increased need of fatty acid transporter to shuttle fatty acids into the mitochondria for beta-oxidation or for the release into the plasma. The observed changes in long-chain AC and free carnitine were recently associated with metabolite levels before and after fasting [216].

In case of energy depletion and glucose shortage, amino acids can be directly converted to glucose. Especially Ala and BCAAs can act as metabolic fuel in the absence of glucose by their degradation [217] and introduction to the TCA cycle via acylcarnitines (Figure 4). The most pronounced and significant changes during the acute treatment occur in the SGLT2I treatment group for isoleucine and butyrylcarnitine (C4-OH) in all observed organs and plasma. C4-OH is a product of Lys or Trp degradation and a strong marker of ketone metabolism. C4-OH plays an important role in the adaption to fasting[218]. The plasma levels of Ile and Leu are also known to be increased during early stats of fasting in which blood glucose levels be decreased [219], [220], whereas Ala levels are decreased in plasma [217], [220] as observed during the acute SGLT2I treatment. The highly effective removal of glucose by the action of the SGLT2-Inhibitor had the same effect as the early state of fasting and therefore led to a systemic increase in Ile and its oxidation. Both effects are reflected by the increase of the respective metabolite levels. In addition, the levels of Leu and Ile as well as acylcarnitines related to AA degradation were found to be increased in plasma of the acute SGLT2I treated mice. In response to the acute SGLT2I treatment, high levels of BCAA and enhanced level of AA related AC reflect an increased protein catabolism in all peripheral organs. Increased protein catabolism as well as enhanced BCAA oxidation reflects effects similar to that observed in the early fasting state.

For the acute combination treatment similar regulation pattern as for the SGLT2I occurred. In addition to the observed effects from the SGLT2I elevation of hydroxylated long-chain AC in the combination treatment group were observed. Increased long-chain hydroxylated AC indicated impairment in β-oxidation and an increased activity of fatty acid hydroxylases like CYP4. This reaction of the organs

is again caused by the decreased glucose levels. The increase in long-chain ACs and the activation of CYP4 during fasting are well described [221], [222].
The observed effects in AC and AA homeostasis during the acute treatment occur mainly in the SGLT2I and combination treated groups and generally mimic the short-term metabolic control during fast depletion of glucose. In general the observed AC and AA homeostasis was found similar to those alterations reported during the early sate of fasting.

Additionally, to AC and AA, [216] described several lysoPCs which are associated with fasting. The effects of the acute SGLT2I and combination treatment reflect findings from this fasting study. In [216], lysoPC16:0 and lysoPC17:0 were found to be decreased in plasma during fasting and increased after food intake. In this thesis, lysoPC.a.C17:0 is significantly reduced in plasma during both treatments. Additionally, various ratios between lysoPC.a.C16:0 and other metabolites indicated a decrease of this metabolite.

It was discussed controversial if periodic caloric restriction are clinically or metabolic beneficial [223]. However, latest results indicate a beneficial effect of periodic caloric restriction to cardiovascular and metabolic disorders [224]. The daily sub-chronic treatment with SGLT2I mimics this effect by depletion of blood glucose levels and changes in lipid and amino acid utilization as described above.

During the 14 day sub-chronic treatment the continuous antidiabetic drug interventions should lead to a normalization of the energy homeostasis disturbed by T2D. Significant changes occur only for metformin and combination treatment. The SGLT2I treatment showed no significant changes on any of the investigated metabolite levels or ratios in any of the organs or plasma. Also the basic readout parameter in T2D, blood glucose levels were found to be not significantly altered. This missing long-term glucose lowering effect of SGLT2I was observed before and was associated with enhanced glucose production triggered by the action of SGLT2I [49], [50].

Finally, the acute SGLT2I treatment mimics the state of fasting or caloric restriction, but sub-chronic therapy had no significant effect on any metabolite level. This goes in accordance with results published from [168], showing that neither blood glucose levels nor HbA1c levels were significantly decreased during sub-chronic treatment. However, [168] showed that sub-chronic SGLT2I treatment improved glycemic control. Additionally, results from caloric restriction studies associated beneficial effects with altered plasma cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels [223], [225], which were not investigated in this thesis and may lead to clinical relevant alterations. In conclusion, there are remaining open questions whether SGLT2I treatment itself improves metabolic health over long-term trials, if there are harmful side-effects due to insufficient caloric availability, or if there an optimal time point for the administration of SGLT2I such as postprandial administration.

4.9.3. Effect on lung

T2D and the metabolic syndrome are disorders related to micro- and macrovascular pathology. The lung itself is a microvascular unit and its function is impaired within T2D. However not much is known about the nature of the relationship between lung function and T2D [226]. Therefore, changes in the metabolic state during antidiabetic treatment specific to lung were investigated.
From the peripheral organs, the lung showed most severe changes in AC homeostasis after sub-chronic metformin and combination treatment. Interestingly, changes in AC levels local to the lung, and no comparable AC pattern was found in plasma. The observed elevated levels of hydroxylated long-chain ACs can indicate changes in long-chain-3-hydroxyacylCoA dehydrogenase activity. As 3-hydroxyacylCoA dehydrogenase is also involved in the degradation of Lys and BCAA, the increase in this amino acids give a second hint for perturbations in the activity of this enzyme class.
The second enzyme class related to the hydroxylation of fatty acids was ω-hydroxylases. The enzymes of CYP family, especially the CYP4A subfamily are known for their capability to hydroxylyse fatty acids from C12-C16. They are activated during intervention with hypolipidemic drugs in rodents and may prevent lipotoxicity [222].
Diabetes related alteration in lung AC content are poorly investigated, and their function and contribution to disease pathology remains unclear. Additionally, the lung specific regulation, activation and substrate specificity of the enzymes involved in AC turnover are not completely understood, leaving a gap in the understanding of their relation to diseases. However, first results underline the individual lipid composition of lung and their local response to pathological conditions [227].

In contrast to the lung, only one hydroxylated AC was found to be significantly increased (C16-OH) in plasma during metformin treatment. Instead, a significant decrease of even chain ACs from C4 to C18 was observed during combination treatment in plasma. This underlies the assumption that regulation of lung AC homeostasis was not driven by uptake of ACs from the blood, but a local, lung specific effect.

4.9.4. Sub-chronic alterations in plasma metabolite homeostasis

Epidemiological evidence has demonstrated a link between T2D, obesity, and distortions of mitochondrial fatty acid oxidation and increased plasma AC levels [141], [142]. The here observed decreases of plasma AC levels during the sub-chronic combination treatment were also associated with improved insulin sensitivity [228]. The observed decrease of AC in plasma is clearly stronger in the combination treatment than in the metformin treated group. Indicating an additionally effect of the combination treatment over the single drug intervention. However, it was shown that plasma AC levels in antidiabetic treated *db/db* and C57BL/6 mice depend strongly on fed or fast state [228]. As here only AC levels after 4 hours of fasting were determined, the results from a fed experiment may look different.

Decrease in plasma AC levels only in part reflects the fatty acid uptake or mitochondrial oxidation rate. This suggests that alteration in AC homeostasis was also related to other mechanisms which may play a role in the effect of the antidiabetic drugs, e.g. PPARα or PGC1α signaling [212], [228].

4.9.5. The BCAA story

Interest in branched chain amino acids (BCAA (leucine, isoleucine, valine)) in diabetes research and treatment highly increased in the last years as they are strongly associated with diabetes [51], [83], [229], [230], used in diabetes therapy [231], proposed as biomarker for diabetes risk or insulin resistance [232]–[234], as well as biomarker for adipogenesis and obesity [235], [236]. These findings are nearly identical to the changes observed in this thesis. The vehicle treated db/db mouse model showed significant elevated plasma levels of all three BCAA compared to the wildtype. However, elevated blood levels of BCAA during insulin resistance and T2D were investigated under static conditions or in epidemiological studies and only few studies are performed of the response of BCAA to acute and sub-chronic antidiabetic intervention [237], [238]. An acute 2 days, multiple high-dose metformin intervention results in enhanced plasma BCAA levels in humans [237]. In contrast, in this work no response of BCAA levels to metformin treatment was observed during acute or sub-chronic treatment. One reason might be the different metformin dosages. One intervention with a single metformin dose per day were administered in this thesis, where in [237] 4 times high-dose metformin were applied at each day.

Significant changes in BCAA were only observed during acute SGLT2I treatment. Ile and Leu were found significantly increased in plasma. Additionally, Ile concentrations were found to be elevates in

all tissues, significant as single metabolite in testis, lung and cerebellum, and for several ratios between Ile and other amino acid in adrenal gland. This results are completely in accordance with [220] who showed a increase in plasma BCAA during the fasting. Additionally, the results from [220] showed that Ile was the most effected BCAA followed by Leu and in a lesser extent Val. Combination treatment nearly abrogates this effect in plasma as well as in tissues. In [237] elevated BCAA levels during acute intervention were implied as signal for improved glycemic control. The increase in BCAA levels, observed during acute SGLT2I treatment, fall together with significant decreased glucose levels. However, it remains unclear if they are a sign of improved glycemic control or of elevated protein degradation. One point against elevated protein degradation is that the elevation of BCAAs levels was not accomplished by increase in other amino acid levels.

As elevated levels of BCAA were associated with insulin resistance and diabetes, the levels should be decreased during a sub-chronic antidiabetic intervention. The results clearly show that there is no such significant effect in any of the sub-chronic treatment groups, neither for circulating BCAA levels nor for tissue specific levels. This indicates that the biochemical pathways involved in the pathophysiology of BCAA in diabetes and obesity are not or less effected by the here applied antidiabetic treatments.

4.9.6. Alpha amino adipic acid – response of a T2D biomarker

An increase in plasma levels of the amino acid alpha aminoadipic acid (alphaAAA) during the sub-chronic combination treatment was observed. Elevated plasma levels of alphaAAA were associated with the development of diabetes and suggested as biomarker for diabetes prediction [239], [240]. It was assumed that the increase in plasma alphaAAA levels in early diabetes is associated to an enhanced activity of β-cells and increased insulin secretion in early diabetes. Treatment with alphaAAA decreases basal glucose level in high-fat and normal diet mice and support insulin secretion. alphaAAA is present in many tissues, but most prominent in pancreas [239]. As pancreatic levels of alpha AAA were not investigated here, only plasma levels were evaluated. The levels of alphaAAA in the other organs do not change significantly which is in accordance to previous findings [239]. However, in testis (which was not investigated in [239]) of combination treated mice changes in alphaAAA concentration equaled this found in plasma. There are no data about alphaAAA action in testis of diabetic subjects, and it remains unclear in which way alphaAAA affect the physiology or endocrine function of testis in diabetes. If the observed increase in plasma alphaAAA levels are related to pancreatic concentrations this may improves β-cell function and insulin levels, and can in part explain the normalization of glucose and HbA1c levels during the combination treatment.

The biochemical pathway behind this effect remains unclear as the alphaAAA pathway itself is not well characterized. Clearly, alphaAAA occurs from the degradation of lysine. Levels of both amino acids were found to be elevated in plasma of the combination treated mice. This goes in accordance with results from prediabetic mice in which Lys and alphaAAA were both elevated in plasma [241]. Further, the alphaAAA/Trp ratio was significantly altered during the combination treatment. This indicates a downstream effect of the alpaAAA metabolism to Trp degradation. AlphaAAA was shown before to alter the Trp degradation pathway [242], but the biochemical function to diabetes remain unclear.

Another correlation for alphaAAA and Lys to acylcarnitine (C12, C12.1) was observed. Metabolism of medium chain acylcarnitines and alphaAAA are linked as defects of acyl-CoA dehydrogensase are correlated to alterations in alphaAAA homeostasis [243]. Trp and AC metabolism are closely linked to diabetes; changes in their homeostasis during combination treatment may positively affect the glucose homeostasis.

4.10. Phosphatidylcholine synthesis and remodeling

In both the acute and the sub-chronic treatment groups changes in lipid/phosphatidylcholine levels were observed.

Alterations by acute treatment were found in plasma, lung and in part in adrenal glands. The phosphatidylcholine (PC) levels were not significantly altered in the other peripheral organs. The most prominent changes were found for SGLT2I and combination treatment in plasma.

During acute combination treatment, the observed effect on PC lipid profile resulted mainly from the treatment with the SGLT2I, since metformin had only a minor impact on PC homeostasis. For the SGLT2I only weak effects on other proteins then the SGLT2 transporter are reported [48], [244] and primary only the decrease in blood glucose levels is observed and therefore this was the main factor driving the changes in PC homeostasis during acute treatment. This single but massive decrease in blood glucose levels caused by the acute SGLT2I and combination treatment led to an fast response in the PC profiles mainly in plasma (SGLT2I and combination treatment) and in part in the lung (combination treatment).

The systemic dropdown of glucose which is the main energy source resulted in higher need of energy in tissues which can be counteracting by providing more lipids as energy source. Fatty acids as energy supply are transported in plasma by different lipoproteins which mainly consist of PCs. This is potentially the cause of the change in plasma lipid profiles. However, no significant effects on total amount of PCs in plasma were observed. Furthermore, these fast PC alterations in plasma do not cause

a significant response to the PC composition or levels in peripheral tissues, except the lung. This indicates that the effects of the acute treatment on PC levels are a fast, primary response to the antidiabetic treatment and that the periphery organs are not affected nor involved in PC level alteration found in plasma.

The metformin induced change from acute treatment in plasma content of PC.aa.32.1 potentially caused by a shift in the syntheses pathways of PC in liver. The CDP-cholin pathway prefers the incorporation of C16:0 fatty acid constituents [149]. The resulting PCs are further built up lipoproteins and are excreted into the blood. These provide a possible explanation for the increase in PC.aa.C32.1 content found in the plasma, as PC.aa.C32.1 should mainly consists of two C16 fatty acids. It remains unclear if this metformin specific alteration occurs in the other treatment and is here just not observed for a low significance or just counteract by changes induced though the different treatments.

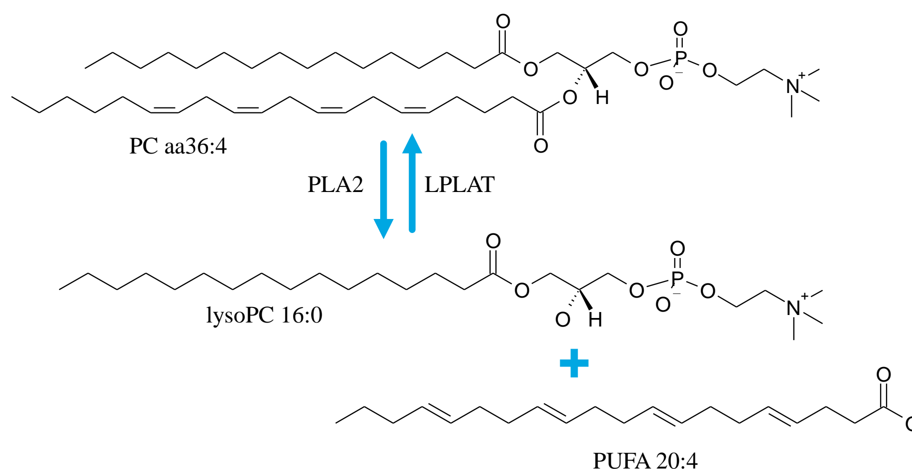

Figure 21: Lipid remodeling during Land's cycle
After the de novo synthesis of phospholipids, individual phospholipids are subjected to the remodeling pathway. Phospholipase A2 (PLA2) releases one fatty acid from the PC, resulting in a free fatty acid and a lysoPC. Lysophospholipid acyltransferase (LPLAT) mediates the reacylation step of lysoPC back into PC. LPLAT can use any fatty acid as substrate but favors PUFAs.

Most PCs in plasma are part of lipoprotein particles. Therefore, the plasma data indicate an alteration in lipoprotein composition in these mice. Neither total amount of PC nor amount of all PCaa or PCae had changed significantly in plasma of sub-chronic treated mice. Therefore, the observed alteration definitely did not come from increased *de novo* synthesis but instead from remodeling of the existing fatty acids as described by [245] (Figure 21). One of the main pathways of lipid remodeling is driven by lysophospholipid acyltransferase and lysophospholipase/transacylase (Figure 21). These two enzymes convert lysophosphatidylcholine (lysoPC) to PC [246]. The alterations in PC composition

goes along with enhanced lysoPC levels in testis, lung, cerebellum and plasma in the sub-chronic combination therapies, indicating an impact on this pathway. Especially CoA-independent transacylase is assumed to be responsible for the accumulation of polyunsaturated fatty acids in ether containing phospholipids [246], given a possible explanation for the observed shift from PCaa to PCae in the sub-chronic combination treatment (cerebellum, lung, plasma) which goes along with an increase in saturation status of the PCs.

It should be mentioned that acyltransferase and transacylase transfer fatty acids between different phospholipid species (phosphtidylcholin, phosphstidylethanolamine, phosphatidylinositol, phosphatidylserine) [246]. In this work only lysoPC and PC levels were analyzed, and for that reason not all possible interaction between lysoPC and other phospholipids can be examined here. Therefore, lysoPC/PC ratios do not necessarily reflect the direct conversion of a distinct lysoPC into a specific PC, but can only reflect the general alteration in fatty acid turnover.

As diacyl-phosphatidylcholines but not acyl-alkyl phosphatidylcholines are essential for the secretion of HDL particles[80], the observed shift from PCaa to PCae may explain the correlation found in [83] between plasma PC and HDL in diabetic patients.

The shift from PCaa to PCae may contribute to the enduring decrease of plasma glucose levels in the sub-chronic combination treated mice. Higher levels of acyl-alkyl-phospholipids were positively linked to insulin sensitivity [83] and lowered in subjects with insulin resistance [248]. The observed plasma glucose lowering effect, which occurs beyond the time of direct drug action during the sub-chronic combination treatment, can be in part explained by the PCae mediated increased insulin sensitivity.

Even if the result are not direct transferable to the human metabolism, results from cohort studies indicating a common pattern in affected metabolites. The increase in diacylphosphatidylcholine (PCaa) (C32:1, C38:3, C40:5) together with a decrease in acyl-alkyl-phosphatidylcholines (PCae) (C34:3, C40:6, C42:5, C44:4, C44:5) were observed in diabetes patients and found to be predictors for T2D [83]. These metabolites were found to be under the most affected PCs by the different antidiabetic treatments and may reflect improvements in the control of T2D in the mice.

Beside the enzymatic conversion of lipids, oxidative processes are a second factor in fatty acid modulation. High blood glucose over a prolonged period is leading to increased systemic oxidative stress. The mammalian brain composition comprises a high amount of unsaturated lipids, making it especially vulnerable to oxidative damage [249]. The results showed that sub-chronic metformin and particular combination treatment markedly increases the concentration of PCs which possesses 4, 5, or 6 double bound species. Cerebellum, lung and plasma were the main sites of action, from which cerebellum exhibit the most pronounced improvement in the levels of very-long-chain,

polyunsaturated lipids. This indicates a stabilization of cerebral and systemic unsaturated lipids by reduction of oxidative stress [250].
A second hypothesis for the changes found in lysoPC and PC is based on the antioxidative properties of the enol ether double bound of ethanolamine and choline plasmalogen [251]. As PCae can be converted to plasmalogens by insertion of a double bound at the sn-1 position the observed increase in PCae are also related to an increased production of plasmalogenes. Plasmalogens markedly prevented the oxidation of PUFAs [252] and potentially responsible for the observed shift to more unsaturated PCs in plasma of combination treated mice, due to lesser oxidation during metformin and combination treatment.

In general, lipids with longer chain length and higher degree of unsaturation offer protection from diabetes, whereas shorter chain length and saturated fatty acid residues can trigger diabetes [253], [254]. The observed shift to the longer chain length and higher degree of unsaturation which was found during the sub-chronic metformin treatment was clearly enhanced in the combination treatment. Beside the observed decrease in blood glucose, the combination treatment may improve protection from diabetes and cardiovascular diseases in a much stronger way than metformin or SGLT2I alone.

It remains unclear which biochemical pathway is responsible for these effects. However, the shift to more unsaturation can be facilitated by enhanced desaturase activity. Desaturases, mainly Δ6 and Δ5 desaturase are depressed during diabetes resulting in a lower content of arachidonic acid [255].
The specific fatty acid composition of the PCs regarding number of carbons and double bounds cannot be determined with the Absolute*IDQ*™ p180 assay. But information of the lipid composition can be drawn in part from the lysoPC content. During the sub-chronic combination treatment the arachidonic acid containing lysoPC.a.C20.4 was increased in lung and cerebellum. The fatty acid 20:4/20:3 ratio, a marker for Δ5 desaturase activity, was found to be decreased in the equivalent lysoPC ratio (of lysoPC.a.C20.3/lysoPC.a.C20.4) in plasma, indicating an activation of the desaturase. Studies on genetic variation in the *FADS1* gene which encodes the Δ5 desaturase showed the influence on glucose metabolism [256]. This added another possible explanation for the observed decrease in blood glucose during the sub-chronic combination treatment.

Composition of PCs varies between tissues as well between species. Mammalian lung generally yields phosphatidylcholine with a high proportion (50%) of dipalmitoylphosphatidylcholine (two C16:0 fatty acids), whereas the distribution of the different PC species vary more in other tissues between the species [257]. The availability of a lipid species can influence the direction or substrate specific action of an enzyme. Therefore, the results observed in a distinct organ in mice are not necessarily transferable to human or other species.
Composition of the diet influences the phospholipid composition of tissues [258] and can be in part responsible for the observed alterations in PC content between wildtype and db/db mice. Both are

administered to the same high-fat diet, but leptin receptor deficiency led to higher food intake in db/db mice and therefore to a raised incorporation of lipids into PC species.

4.11. Brain specific phosphatidylcholine regulation by histamine

The sub-chronic combination treatment resulted in a cerebellum specific alteration of Histamine/PC ratios. Histamine release is linked to altered biosynthesis of phosphatidylcholine [259], and it stimulates the synthesis of phosphatidylcholine from phosphatidylethanolamine by phospholipid methyltransferases in the brain [260]. As phospholipid methyltransferases activity is affected by diabetes [261], the results indicate an influence of the antidiabetic combination therapy to this pathway. However, the observed effects are only small and different co-factors are involved in this process, leaving some open questions.

4.12. Brain specific carnosine homeostasis

The dipeptid carnosine have been found in the brain of different mammalians, including mouse and human [262]. It shows anti-oxidative and anti-glycating properties. Therefore, it can protect proteins and lipoproteins from oxidation and glycation, especially during periods of high glucose as in diabetes [263]. The degradation of carnosine into β- alanine and histidine is facilitated by carnosinase (CN) (Figure 22). Hyperglycemic state enhances CN secretion as well as its activity [264]. An increase in CN activity results in early onset of diabetes in a db/db mouse model. If carnosine was supplemented to the same db/db mice, glucose metabolism was improved, insulin levels were elevated, and onset of diabetes delayed [265]. Studies in human have shown that carnosine levels are lower in humans with diabetes. Additionally, studies in rat and human support the assumption that carnosine have protective effects against complications associated with diabetes [266]. Furthermore, results from [267] showed a carnitine specific regulation pathways in the brain and they suggested carnosine as potential therapeutic for neuronal disorders.

The data from the sub-chronic combination treatment strongly indicated a reduction in CN activity. Carnosine was found enhanced in cerebellum samples of the combination treated mice. This was accomplished by a decrease in cerebral histamine levels. Carnosine is converted to histidine by CN, and further to histamine. Therefore, decreased carnosine degradation results in decreased histamine concentrations. This indicates a brain specific protective effect of the combination treatment.
The observed enhanced carnosine levels were brain specific. Only weak, not significant alterations

were observed in plasma of the combination treated group. Therefore, it remains unclear in which extent this effect can affect the systemic response to carnitine. However, the combination treatment is related to improved glycemic control, compared to the single drug treatments. Therefore, systemic carnitine homeostasis can be one of the factors explaining this improvement.

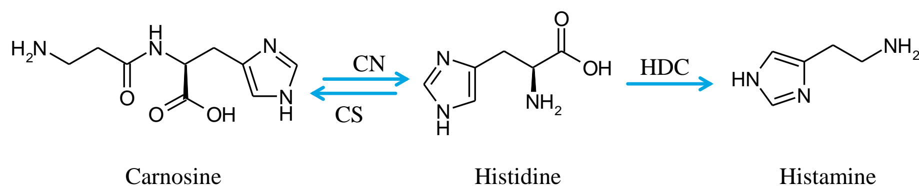

Figure 22: Carnosine metabolism.
Carnosinase (CN) catalyses the degradation of carnosine into histidine and β-alanine. The reverse reaction is catalysed by carnosine synthse (CS). Histidine decarboxylase (HDC) catalyzes the reaction from histidine to histamine.

As stated in chapter 4.7, eNOS activity and NO production were putatively enhanced in the sub-chronic treatment. It was shown that NO can S-nitrosylate CN and decreases carnosine degradation in db/db mice [268]. Therefore, enhanced systemic NO concentration, led to lower CN activity and the observed higher carnosine levels. Thus, higher systemic Cit levels and enhanced brain specific carnosine levels increase the scavenging of reactive oxygen species which may positively affect cerebral functions.

4.13. Lysophosphatidylcholine

Lysophosphatidylcholines (lysoPC) are important lipid signal molecules interacting with diverse biochemical pathways and are involved in inflammation processes. They are major components of oxidized low-density lipoproteins and involved in the systemic transport of fatty acids and choline [269].

Alterations observed for the metabolite class of lysoPC, were in part explained by the above described changes in PC homeostasis involving the PCaa to PCae convertion by lysophospholipase/transacylase and acyltransferase, which involves lysoPC as intermediate product. In general, lysoPCs result from partial hydrolysis of phosphatidylcholines by phospholipase A2, which remove the fatty acid at the sn-

2 position, mostly a PUFA from the PC. The mobilized free fatty acids itself are potent cellular signaling molecules [270], but were not investigated in this thesis.

Since the sn-1 is generally occupied by a C16, C18 or C18:1 fatty acid, the resulting lysoPC is mostly a lysoPC(16:0), lysoPC(C18:0), or lysoPC(18:1). As expected, these are the most significant effected lysoPC species found altered in most of the observed samples and treatments. However, the activity of cytosolic phospholipase A1 (PLA1) removes the fatty acid from the sn-1 position, resulting in lysoPC with C18 or longer polyunsaturated fatty acid chains [271]. The observed tissue specific increase in lysoPC(20:4) in lung and cerebellum during the sub-chronic combination treatment indicated a tissue specific activation of PLA1 during the combination treatment.

In general, decreased levels of circulating lysoPCs were observed in obese and diabetic rodent and humans. In plasma, the most decreased lysoPCs were lysoPC(18:0), lysoPC(18:2), lyosPC(20:0), and lysoPC(20:4) which were strongly related to the diabetic phenotype [84] and lysoPC(18:1) which were associated with obesity [84], [272]. Additionally, lysoPC 18:2 in serum were found inversely related to type 2 diabetes risk [83].

The elevation of lysoPC(18:1) and lysoPC(18:2) specifically during sub-chronic combination treatment occurred not only in plasma, but additionally in lung, cerebellum, and testis (testis only lysoPC(18:2)). This indicates that these biomarkers were affected also in peripheral tissues. The specific increase during the sub-chronic combination treatment suggests a beneficial effect of the combination treatment over the single drug intervention, implying an improvement of the diabetic state.

In the sub-chronic treatment groups lysoPC levels were found to be elevated, including a remarkable amount of unsaturated lysoPCs. The unsaturated lysoPCs are more susceptible to oxidation processes, therefore their accumulation indicating a lesser oxidative environment in the sub-chronic combination treated mice. Diabetes accompanied with increased oxidative stress. The increased level of lysoPC in testis, lung, cerebellum and plasma indicates a systemic reduction of oxidative stress by the sub-chronic combination therapy.

In addition to the elevation of known plasma markers and lysoPC(20:4), lung and cerebellum showed tissue specific elevation of lysoPCs during the sub-chronic combination treatment. Both tissues exhibit increased level of lysoPC(16:0). Additionally, lysoPC(17:0) were elevated in lung, and lysoPC(16.1) in cerebellum. This tissue specific pattern reflects the different lipid compositions between the organs and indicates that the alteration of lipid turnover effect the enzymes or the oxidation status not only on a systemic level but also site-specific.

The acute therapeutic intervention with the different drugs resulted in a quit opposite picture. At first, organs are less affected by the different treatments and only the systemic levels of lysoPCs were

altered by the combination treatment. In contrast to the sub-chronic treatment, systemic lysoPC levels were diminished. As the alterations only occur in the combination treatment, they are a combined result of short-term metformin action and global energy state. As shown in 4.5, the acute metformin action can be quite opposite to the sub-chronic effects and acutely may trigger different biological pathways. The SGLT2I changes the energy and nutritional status of the body. Findings from [273] implicate that the energy status as well as fasting influences lysoPC levels. Together, the action of metformin and SGLT2I can result in the observed decrease of lysoPCs in plasma.

The results from sub-chronic combination treatment can lead to the assumption that PLA2 is an effective target for therapeutic intervention. However the lipoprotein-associated PLA2 (Lp-PLA2) activity is positively associated with insulin resistance and diabetes [274], as well as with cardiovascular disease [275]. However, the role of PLA2 remains controversial [275], linking its lipoprotein associated activity to divers inflammatory effects [85]. However, studies linking enhanced levels of lysoPC to pathological effects observed enhanced level of lysoPC(16:0) or lysoPC(18:0) [85] but not of lysoPC(18:1) [276]. Others investigate only global lysoPC levels [277]. In this thesis mainly mono- or polyunsaturated lysoPCs were elevated and linked to improved metabolic control. It can be concluded that not the specific action of PLA2 but additionally the global lipid composition in cells or lipoproteins play an important role in the downstream effects of PLA2 action.

4.14. Sphingomyelin

The largest number of affected SM levels or PC/SM ratios was found in plasma. SM levels in organs were generally not affected by the antidiabetic treatment. Only testis and lung of sub-chronic combination treated mice exhibit individual changes.

The observed ratios between PC and SM in plasma samples indicate a close association between this two lipid classes. As they do not have a close connection in their biosynthesis pathways; another mechanism should be responsible for this interaction. A strong alteration in lysoPC and PC levels was observed especially during the combination treatment, indicating a higher activity of PLA2. As a result of increased PLA2 activity a higher amount of free fatty acids should be available. These free fatty acids may act as signaling or regulatory molecules. It is described that free PUFAs and especially 20:4 (eicosatetraenoic acid), and in part 20:5 (eicosapentaenoic acid), and 22:6 (docosahexaenoic acid) or their bioactive metabolites had the capacity to stimulate neutral sphingomyelinase activity [278], [279]. Sphingomyelinase catalyzes the hydrolysis of sphingomyelin to ceramide [280] and thereby lowers the SM concentration. A variety of studies have shown that sphingomyelinase activity is related to artherosclerosis and heart failure as reviewed by [281]. The changes in SM levels in plasma

maybe arises from the altered activity of sphingomyelinase that has been reported to be elevated in T2D [282].

Since PLA2 liberates the fatty acid from the sn-2 position (mostly a PUFA), and most PC contain a C16:0, C18:0, or C18:1 chain on the sn-1 position, PC with 36 to 40 carbon atoms in their side chains and 4 or more double bonds can be sources of C20:4, C20:5, or C22:6 release. Most of the plasma PC/SM ratios include such PC species. This enhancement of free PUFA levels can result in a higher activation of sphingomyelinase and therefore can lead to a decrease in SM concentration.
However, the sub-chronic treatment with metformin alone or with the combination therapy causes no significant alteration in total SM concentration, but only a decrease for single SM in lung (SM(16:1)) and in plasma (SM(OH)22:1).
A number of factors is associated with alter ceramide synthesis. Mainly long-chain saturated fatty acids and not unsaturated fatty acids are required for the formation of the sphingoid backbone [283]. The observed shift to more unsaturated fatty acids was sufficient to diminish the formation of ceramides from SMs. Additionally, metformin itself enhances AMPK activity and would likely promote lipid metabolism by regulating acetyl CoA carboxylase, resulting in lesser formation of ceramids [284]. These mechanisms can counteract the activation of sphingomyelinase and are an explanation for the unchanged total SM level during the sub-chronic treatment.

The observed significant shift from hydroxy sphingomyelins (SM(OH)) to SM could be related to diabetes. Only a few studies are available accounting the role of SM(OH) in diabetes. In diabetic pigs the amount of SM(OH) raises with age and progression in diabetes [285]. Additionally, this study linked plasma SM(OH)22:1 and in part SM(OH)22:2 levels to β-cell mass and insulin response and showed alteration of genes involved in SM metabolism.
One protein involved in the hydroxylation of fatty acids, ceramides and sphingolipids is the fatty acid 2-hydroxylase (FA2H). Depletion of FA2H has been connected to insulin resistance and impaired glucose uptake in adiopocytes [286]. Therefore, the decrease of systemic SM(OH)22:1 levels together with the general decrease in SM(OH) during metformin and combination treatment indicates an improved FA2H activity and can be linked to improved β–cell function and insulin response.

SMs are a major compartment of lung surfactant. The lung specific response to the sub-chronic combination treatment resulted in a decrease of SM(16:1). This metabolite was associated before with the risk for diabetes [83]. However, the tissue specific impact remains unclear.

The acute treatment resulted in less alteration in SM pathway. Only in plasma of SGLT2I and combination treated mice an appreciable amount of alteration were observed. The fast and intense decrease of blood glucose caused by the SGLT2I led to a significant alteration in PC/SM levels.

Interestingly, plasma PCae/SM and PCaa/SM ratios exhibit different regulation pattern during acute metformin and combination treatment. During the metformin intervention, PCae with 34 to 40 carbons and 4 or more double bounds are positively correlated to SM levels, while the same PCaa levels were negatively correlated to SM in the combination treatment. SGLT2I had no effect on these ratios. Therefore, the acute action of the combination treatment resulted in a quite unique response that cannot be explained by metformin or SGLT2I action. The specificity of this alteration emphasizes a combination treatment specific regulation. However the underlying mechanisms and the long-term impact remain unclear.

4.15. Specific metabolite response to sub-chronic combination treatment

As explained in detail in the chapters above, most of the observed changes in the combination treatment can be linked to improved metabolic control. Interestingly, two of these biomarkers occur not only in plasma but found altered in the tissues. These are lysoPC(18:1) and lysoPC(18:2). As alteration of both lysoPC levels occur in plasma and peripheral tissues it points to an alteration in global lipid composition. Their response to antidiabetic treatment occurs on different sites of the body making pathways involved or affected by lysoPC homeostasis an interesting target for new drugs.

PCs are the second fraction of metabolites altered by the combination treatment. The levels of specific PC were found enhanced mainly in plasma and cerebellum. PCs are the major component of cellular membranes and represent a major fraction of the plasma lipidome. Changes in PC content can lead to changes in membrane associated protein activity. In plasma, alteration of PC levels effect the composition of lipoproteins and can result in altered cholesterol transport. They are important for the hepatic secretion of lipoproteins.
Interestingly, the concentrations of three PCs were lowered in lung. This finding is contrary to the observed results from the systemic plasma levels. As PCs are major compartments of pulmonary surfactant they may have their own regulation mechanism. The lung specific increase of odd chain fatty acid containing lysoPC(17:0) and an odd-chain AC (C5.1) indicated a tissue specific fatty acid and lipid profile.

All metabolites shown in Table 9 and Figure 18 are singular affected only by the sub-chronic combination treatment. The range of this thesis is limited to metabolomics, and only assumptions of the underling mechanisms are possible. However, these metabolites are all interesting for future research of the systemic and organ specific pathology of T2D and the metabolic syndrome, and as novel drug targets.

4.16. What are the benefits from combination drug treatment?

In contrast to the acute antidiabetic intervention, the sub-chronic SGLT2I intervention did not cause substantial effects on hexose, lipid or amino acid metabolism in plasma or peripheral tissues, whereas the sub-chronic treatment with metformin caused more alteration in plasma, cerebellum and lung. The daily reduction of blood glucose by SGLT2I did not reduce HbA1c levels in the here investigated mice [168] and did not change the causes of diabetes or metabolic syndrome. Also metformin alone fails in normalizing blood glucose levels and did not improve HbA1c. However, substantial changes occurred in the amino acid and acylcarnitine pathways of lung and plasma. Interestingly the sub-chronic combination treatment causes more and stronger effects in all of the examined organs. At first, the two main readout parameters for successful antidiabetic interventions; blood glucose and HbA1c, were only significant normalized in the combination treatment, implicating a far-reaching effect of this treatment. Since the last administration of the antidiabetic drugs in the sub-chronic treatment were 18 hours ago and their half-life were considered to be between two and six hours, observed changes can reflect long lasting effects that indicates a systemic adaption or permanent change in affected pathways, especially in the combination treatment.

The metabolic pattern found specifically in the combination treatment can be the result or the trigger for the observed improvement in glucose homeostasis. For plasma, biomarkers for diabetes are well described and found generally improved in the combination treatment. For the peripheral tissues the relation to diabetes of most of the metabolites is not shown yet.

In summary, the results from this thesis led to the assumption that the sub-chronic combination treatment causes biological relevant alteration in the hydroxylation status of fatty acids and SM, regulation of PC turnover, AC homeostasis, and Cit and alphAAA levels. All of these were before associated with diabetes, the metabolic syndrome or related diseases as cardiovascular diseases. Tissue specific alteration in histamine, MetSO, and PC pointing to new poorly investigated side effects of diabetes and the therapeutic intervention. They are all targets to further improve metabolic health in a systemic manner.

4.17. Statistical relevance or biological relevance

Very often, results from metabolomics measurements are used to screen for perturbations in a biochemical pathway of interest under certain conditions. Depending on the aim of the study, the

choices of the statistical method affect the outcome of the data analysis [287], [288]. Observing differences in metabolite concentration or ratios, a statistically significant difference is required in order to present a metabolite of interest. In the biological context a statistical significance may be not relevant for the condition of interest or has no real influence on the pathway of interest. Systematic changes in metabolites belonging to one pathway or in ratios of interrelated metabolites can give a clue about the biological relevant alteration under a specific condition or may be act as biomarkers in further analyses.
This thesis showed the power of metabolite ratios to identify complex interactions that goes beyond the direct implication in a single biochemical pathway. The correlations on metabolite level led to new insights in the regulation of diabetes associated mechanisms and help to explain the mode of actions of antidiabetic drugs.

4.18. Limitations

While the metabolite profile only provide a snapshot of the human metabolome, different time points were necessary to follow the metabolite dynamic during the antidiabetic intervention. Moreover, the sub-chronic treatment is restricted to one time point. A prolonged study can give more insights into the long-term effects of drug interventions.

Action of metformin depends on the concentration of the drug and metformin pharmacokinetics is altered by SGLT2I. Therefore, observed differences between the metformin and combination treatment can be in part arise from the different metformin concentration.

The animals in this study were fed with a high-fat diet. As the diet itself strongly influences many of the here observed factors, some of the observation may be diet specific.

This is no association study; it does not prove the reliability of one or several metabolites or ratio as biomarker for diabetes or successful drug intervention. The thesis focuses only on the affected biochemical pathways in diabetes and diabetic drug intervention.

The here presented pathways found to be interesting or reliable. However, this work cannot finally explain the origin of the found alterations. The main reason for this is the involvement of metabolites in different pathways, each with it specific cellular regulation and signaling processes. Further deep thrilling studies may explain the underlying mechanisms.

4.19. Conclusion

The results presented in this thesis have demonstrated that targeted metabolomics profiling can allow deeper insights into basic biochemical and cellular processes related to diabetes and antidiabetic drug intervention.

Considering the complexity of biochemical alterations caused by T2D and metabolic syndrome, as well as the large number of site specific pathological events, no single treatment can completely improve all of them. The approach of combined administration of drugs which target different pathological mechanisms is a good step towards the recovery of global metabolic body homeostasis.

In fact, most of the observed changes are predictive biomarkers for T2D or related diseases. The observed metabolic pattern can give insight into specific pathways that are affected by the combination treatment and thereby allow the identification of novel systemic and site specific therapeutic targets.

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6. Appendix

6.1. Metabolic perturbation induced by drug treatment

6.1.1. Acute treatment (4 hours)

Metformin

Metformin Testis 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Cit		2.471(0.298,4.644)	0.04613		-2.03
C3		-0.252(-0.54,0.035)	0.06576		2.47
C2	**C3**	18.831(-0.002,37.664)	0.04613	5.5	-1.83

Metformin adrenal gland 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

Metformin lung 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Cit		7.302(2.557,12.047)	0.00284		-3.12
C16:1	**C4**	-0.215(-0.41,-0.02)	0.0059	491.1	2.22
C16(OH)	**C4**	-0.249(-0.475,-0.023)	0.00615	99.1	2.45

Metformin cerebellum 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

Metformin plasma 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Cit		39.54(8.235,70.845)	0.0002		-2.13
C16(OH)		-0.008(-0.014,-0.001)	0.00151		4.3
C18:1(OH)		-0.005(-0.011,0.001)	0.01506		2.58

lysoPC17:0		0.415(-0.183,1.013)	0.02963		-1.11
C3		-0.3(-0.693,0.093)	0.03173		1.29
PCae36:2		1.545(-0.671,3.761)	0.03888		-1.35
PCae38:2		1.102(-0.584,2.788)	0.05		-1.16
C14:1	**C16(OH)**	2.663(1.085,4.241)	0.0002	896.7	-2.95
C14	**C2**	-0.001(-0.002,0)	0.00682	396.2	1.87
C16	**C18**	-0.634(-1.398,0.13)	0.0147	289.5	2.88
C14	**C18**	-0.214(-0.456,0.029)	0.01059	217.7	2.39
C18:1	**C3**	0.195(0.022,0.368)	0.001	138.9	-1.85
Glu	**Orn**	-0.597(-1.113,-0.08)	0.00091	1982	3.51
Gln	**Phe**	-2.09(-4.193,0.014)	0.00457	320.5	2.47
Gln	**Tyr**	-3.121(-6.492,0.251)	0.0053	262.5	2.92
Gln	**Ser**	-1.668(-3.626,0.289)	0.01158	90.9	2.17
Gln	**His**	-2.211(-4.725,0.302)	0.01521	63.6	1.69
PCaa32:1	**PCae38:0**	-0.194(-0.403,0.015)	0.00544	2202.6	2.64
PCaa32:1	**PCaa34:3**	-0.028(-0.059,0.003)	0.00805	1302	2.7
PCaa32:1	**PCaa36:5**	-0.08(-0.168,0.009)	0.00894	939.9	2.14
PCaa32:1	**PCaa38:6**	-0.004(-0.008,0.001)	0.01282	686.5	2.02
PCaa32:1	**PCaa40:4**	-0.131(-0.284,0.021)	0.01544	512.7	1.99
PCaa36:5	**PCaa40:5**	0.092(-0.03,0.213)	0.02187	271.4	-1.57
PCaa32:1	**PCaa38:4**	-0.002(-0.004,0)	0.02288	245.1	1.69
PCaa32:1	**PCae40:4**	-0.302(-0.65,0.046)	0.01435	233.5	2.3
PCaa38:4	**PCaa40:5**	3.871(-1.392,9.134)	0.02789	183.6	-2.01
PCaa32:1	**PCae38:3**	-0.461(-0.964,0.041)	0.00574	163.7	2.68
PCaa32:1	**PCae32:1**	-1.282(-3.017,0.452)	0.03036	130.1	3.01
PCaa40:5	**PCae38:3**	-1.796(-3.824,0.231)	0.0075	113.9	3.4
PCaa32:1	**PCae40:2**	-1.017(-2.43,0.396)	0.03374	86.9	5.84
PCaa32:1	**PCae38:4**	-0.104(-0.237,0.029)	0.03046	85.8	2.24
PCaa32:1	**PCae42:4**	-2.198(-5.249,0.852)	0.03607	71	1.65
PCaa40:5	**SM18:1**	-1.027(-1.903,-0.151)	0.00129	18108.1	2.98
PCaa40:5	**SM16:1**	-0.21(-0.51,0.089)	0.04938	89.3	3.74
PCae34:3	**SM16:0**	0.003(-0.001,0.008)	0.01741	67.9	-1.87
PCaa32:1	**SM18:1**	-0.284(-0.739,0.171)	0.07111	58	1.13
lysoPC18:0	**PCaa40:5**	3.256(0.26,6.251)	0.00215	309.7	-2.76
C16(OH)	**PCae38:3**	-0.007(-0.011,-0.003)	0.0002	738.6	6.06
C16(OH)	**PCaa36:3**	0(0,0)	0.0002	734.1	7.65
C16(OH)	**PCae36:3**	-0.007(-0.012,-0.002)	0.0002	131.4	6.84
C16(OH)	**PCaa38:3**	0(0,0)	0.0002	127.2	4.37
C18:1(OH)	**PCaa36:3**	0(0,0)	0.00063	123.1	3.11
C16(OH)	**lysoPC18:0**	0(0,0)	0.0002	6603.3	8.4
C16(OH)	**lysoPC16:0**	0(0,0)	0.0002	644.6	8.87
C16(OH)	**lysoPC17:0**	-0.007(-0.011,-0.002)	0.0002	233.7	9.44
C18:1(OH)	**lysoPC18:0**	0(0,0)	0.00036	233.1	3.1
C16(OH)	**lysoPC16:1**	-0.003(-0.004,-0.001)	0.0002	176.1	4.17
C18:1(OH)	**lysoPC16:0**	0(0,0)	0.00063	106.3	3.27
C16(OH)	**SM18:1**	-0.006(-0.009,-0.003)	0.0002	991.8	5.66
C16(OH)	**SM24:1**	-0.001(-0.001,0)	0.0002	205.9	4.16

SGLT2-Inhibitor

SGLT2-Inhibitor testis 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Ile		-15.47(-28.516,-2.424)	0.01074		2.44
C3-DC(C4-OH)		-0.381(-0.826,0.064)	0.01354		1.56
Sum of hexoses		238.13(-9.75,486.01)	0.01354		-2.1
C5-DC(C6-OH)		-0.038(-0.088,0.012)	0.01725		1.53
C3-DC(C4-OH)	**Sum of hexoses**	-0.004(-0.007,-0.001)	0.00086	1493.9	9.34
C5-DC(C6-OH)	**Sum of hexoses**	0(-0.001,0)	0.00507	127	9.96
C3-DC(C4-OH)	**C8**	-1.913(-3.642,-0.184)	0.00922	31536	2.23
C3-DC(C4-OH)	**C5-M-DC**	-23.14(-45.92,-0.358)	0.01149	14566.6	2.48
C3-DC(C4-OH)	**C5:1**	-16.131(-34.729,2.467)	0.01362	11520.2	1.56
C5-DC(C6-OH)	**C8**	-0.187(-0.382,0.008)	0.01074	10254.1	2.28
C3-DC(C4-OH)	**C3-OH**	-6.833(-13.482,-0.184)	0.0156	8755.9	2.74
C5-DC(C6-OH)	**C5-M-DC**	-2.349(-5.092,0.394)	0.01328	8280.4	2.6
C3-DC(C4-OH)	**C5-OH(C3-DC-M)**	-0.868(-1.65,-0.086)	0.01074	2579.9	2.1
C3-DC(C4-OH)	**C6:1**	-14.444(-29.83,0.943)	0.01706	1361.8	2.08
C3-DC(C4-OH)	**C9**	-48.15(-101.663,5.362)	0.02432	1025.6	1.92
C3-DC(C4-OH)	**C6(C4:1-DC)**	-0.794(-1.741,0.153)	0.01725	616.9	1.89
C5-DC(C6-OH)	**C5-OH(C3-DC-M)**	-0.084(-0.18,0.012)	0.01675	393.5	2.14
C5-DC(C6-OH)	**C6:1**	-1.377(-3.188,0.434)	0.03882	231	1.92
C5-DC(C6-OH)	**C6(C4:1-DC)**	-0.077(-0.178,0.025)	0.02853	214	1.94
C3-DC(C4-OH)	**C4:1**	-16.27(-31.51,-1.019)	0.01706	167	2.04
C3-DC(C4-OH)	**C7-DC**	-6.434(-12.804,-0.064)	0.01675	129.9	1.97
C3-DC(C4-OH)	**Glu**	0(0,0)	0.00719	377018.3	10.25
C5-DC(C6-OH)	**Glu**	0(0,0)	0.00719	146460.7	6.93
C3-DC(C4-OH)	**Sarcosine**	-0.102(-0.204,0)	0.01074	27321.7	2.01
C3-DC(C4-OH)	**DOPA**	-2.615(-4.82,-0.41)	0.01074	13329.6	12.72
C5-DC(C6-OH)	**Sarcosine**	-0.01(-0.021,0.001)	0.01384	10035.2	1.91
C3-DC(C4-OH)	**Putrescine**	-0.227(-0.436,-0.017)	0.01074	8818.7	2.42
C5-DC(C6-OH)	**Putrescine**	-0.022(-0.043,-0.002)	0.01074	7996.4	2.36
C5-DC(C6-OH)	**DOPA**	-0.228(-0.443,-0.014)	0.01351	3808.5	14.58
C3-DC(C4-OH)	**Creatinine**	-0.004(-0.01,0.002)	0.0269	2132.8	1.27
C3-DC(C4-OH)	**Cit**	-0.159(-0.336,0.019)	0.01074	1669	3.67
C5-DC(C6-OH)	**Cit**	-0.016(-0.032,0)	0.01074	1469.9	3.57
C3-DC(C4-OH)	**Pro**	-0.003(-0.008,0.002)	0.03252	1354.8	1.14
C3-DC(C4-OH)	**Serotonin**	-4.74(-10.624,1.145)	0.02152	1325	1.53
C5-DC(C6-OH)	**Creatinine**	0(-0.001,0)	0.0437	949.5	1.25
C5-DC(C6-OH)	**Serotonin**	-0.464(-1.128,0.201)	0.03252	585.3	1.47
C3-DC(C4-OH)	**Gly**	0(-0.001,0)	0.0367	218.9	1.24
C4:1	**Cit**	-0.002(-0.006,0.001)	0.01706	167.3	3.38
Putrescine	**Spermidine**	0.081(-0.033,0.195)	0.03437	123.5	-1.28
C3-DC(C4-OH)	**PCae38:0**	-0.967(-2.016,0.082)	0.01362	10169.3	1.97

C3-DC(C4-OH)	**PCae40:2**	-6.971(-13.541,-0.402)	0.01074	9138.1	2.66
C3-DC(C4-OH)	**PCaa26:0**	-0.835(-1.833,0.162)	0.01362	7892.3	1.57
C3-DC(C4-OH)	**PCaa24:0**	-9.118(-18.457,0.222)	0.01384	7848.1	2.05
C3-DC(C4-OH)	**PCae44:4**	-2.61(-5.636,0.416)	0.0156	7622.9	1.96
C3-DC(C4-OH)	**PCaa40:1**	-2.392(-5.185,0.402)	0.01675	7029.7	1.67
C3-DC(C4-OH)	**PCae42:0**	-0.977(-2.162,0.209)	0.01615	6590.2	1.61
C3-DC(C4-OH)	**PCaa36:5**	-0.355(-0.776,0.067)	0.01706	5091.9	1.84
C5-DC(C6-OH)	**PCae38:0**	-0.096(-0.211,0.019)	0.01718	4843.7	1.94
C3-DC(C4-OH)	**PCae38:3**	-2.344(-5.286,0.598)	0.01744	4801.1	2.55
C3-DC(C4-OH)	**PCae34:3**	-2.668(-5.681,0.346)	0.01675	4383.1	2.17
C3-DC(C4-OH)	**PCaa32:3**	-13.92(-29.783,1.942)	0.01706	3944.3	2.05
C5-DC(C6-OH)	**PCae44:4**	-0.255(-0.578,0.068)	0.01894	3791	1.91
C3-DC(C4-OH)	**PCaa34:3**	-0.415(-0.922,0.091)	0.02122	3400.9	1.7
C5-DC(C6-OH)	**PCae42:0**	-0.097(-0.228,0.033)	0.02048	3120	1.59
C5-DC(C6-OH)	**PCaa26:0**	-0.084(-0.197,0.03)	0.01842	2967.4	1.56
C3-DC(C4-OH)	**PCaa36:6**	-2.731(-6.175,0.713)	0.0215	2938.9	1.84
C5-DC(C6-OH)	**PCaa24:0**	-0.903(-1.93,0.123)	0.01914	2927.4	1.95
C5-DC(C6-OH)	**PCaa34:3**	-0.04(-0.09,0.009)	0.02234	2861.1	1.68
C5-DC(C6-OH)	**PCaa36:5**	-0.035(-0.079,0.009)	0.02191	2686.6	1.81
C3-DC(C4-OH)	**PCae42:2**	-0.498(-1.098,0.102)	0.01912	2544.6	1.99
C3-DC(C4-OH)	**PCae42:5**	-1.183(-2.761,0.396)	0.01842	2454.6	1.38
C3-DC(C4-OH)	**PCae36:0**	-3.631(-8.091,0.828)	0.01718	2323.3	2.26
C5-DC(C6-OH)	**PCaa40:1**	-0.243(-0.577,0.092)	0.0259	2279.2	1.68
C3-DC(C4-OH)	**PCaa34:4**	-1.082(-2.464,0.3)	0.02106	2137.7	1.6
C5-DC(C6-OH)	**PCae34:3**	-0.259(-0.579,0.06)	0.02135	2094.7	2.13
C5-DC(C6-OH)	**PCaa36:6**	-0.266(-0.605,0.073)	0.02546	2053.8	1.78
C3-DC(C4-OH)	**PCaa42:0**	-14.719(-35.029,5.592)	0.02432	2030.2	1.53
C3-DC(C4-OH)	**PCaa38:6**	-0.028(-0.066,0.01)	0.0269	1974.8	1.65
C3-DC(C4-OH)	**PCaa36:0**	-0.379(-0.897,0.139)	0.02432	1937.1	1.55
C3-DC(C4-OH)	**PCae36:5**	-0.33(-0.77,0.11)	0.02545	1884.9	1.7
C3-DC(C4-OH)	**PCaa32:2**	-1.989(-4.809,0.831)	0.0259	1858.9	1.41
C3-DC(C4-OH)	**PCae44:5**	-6.683(-15.092,1.726)	0.02772	1834	1.85
C3-DC(C4-OH)	**PCae30:1**	-6.666(-15.289,1.957)	0.0269	1832	2.52
C3-DC(C4-OH)	**PCaa38:0**	-1.06(-2.35,0.231)	0.01992	1821.9	1.74
C3-DC(C4-OH)	**PCae44:3**	-1.297(-2.843,0.248)	0.02165	1669.2	1.98
C3-DC(C4-OH)	**PCaa34:2**	-0.026(-0.062,0.009)	0.02703	1584.7	1.61
C3-DC(C4-OH)	**PCaa36:4**	-0.006(-0.014,0.002)	0.02929	1491.5	1.56
C5-DC(C6-OH)	**PCae38:3**	-0.229(-0.548,0.089)	0.03195	1458.1	2.39
C5-DC(C6-OH)	**PCaa34:4**	-0.105(-0.248,0.037)	0.02684	1254.8	1.55
C5-DC(C6-OH)	**PCaa32:3**	-1.415(-3.359,0.528)	0.02807	1218.6	2
C5-DC(C6-OH)	**PCaa34:2**	-0.003(-0.006,0.001)	0.03174	1203	1.54
C5-DC(C6-OH)	**PCaa38:6**	-0.003(-0.007,0.001)	0.03516	1197.3	1.58
C5-DC(C6-OH)	**PCaa36:0**	-0.037(-0.091,0.016)	0.03137	1167.4	1.5
C5-DC(C6-OH)	**PCae36:5**	-0.032(-0.077,0.013)	0.03308	1123.9	1.65
C3-DC(C4-OH)	**PCae36:3**	-1.53(-3.825,0.766)	0.03839	1074.1	2.32
C3-DC(C4-OH)	**PCae38:6**	-0.046(-0.112,0.019)	0.03662	1033	1.6
C3-DC(C4-OH)	**PCaa36:2**	-0.052(-0.121,0.016)	0.02675	1028.1	1.72

C5-DC(C6-OH)	**PCae42:2**	-0.049(-0.113,0.016)	0.02973	1010.2	1.92
C3-DC(C4-OH)	**PCae40:6**	-0.51(-1.2,0.179)	0.03124	958.6	1.84
C5-DC(C6-OH)	**PCae42:5**	-0.117(-0.293,0.058)	0.02925	941.2	1.34
C5-DC(C6-OH)	**PCaa36:4**	-0.001(-0.001,0)	0.03839	937.5	1.51
C3-DC(C4-OH)	**PCaa42:5**	-1.711(-4.163,0.74)	0.03522	922.3	1.71
C3-DC(C4-OH)	**PCae40:4**	-0.933(-2.073,0.207)	0.02542	921.5	2.01
C3-DC(C4-OH)	**PCae38:1**	-10.746(-25.744,4.253)	0.03352	822.4	1.74
C3-DC(C4-OH)	**PCaa42:6**	-0.609(-1.341,0.122)	0.01744	784.2	1.75
C5-DC(C6-OH)	**PCaa32:2**	-0.194(-0.489,0.102)	0.04242	778.6	1.34
C5-DC(C6-OH)	**PCae44:5**	-0.65(-1.555,0.255)	0.04811	767.9	1.72
C3-DC(C4-OH)	**PCaa36:3**	-0.034(-0.084,0.016)	0.03781	734	1.46
C5-DC(C6-OH)	**PCae44:3**	-0.126(-0.29,0.038)	0.03358	722.8	1.93
C5-DC(C6-OH)	**PCaa38:0**	-0.103(-0.24,0.035)	0.03192	710.7	1.66
C3-DC(C4-OH)	**PCae38:5**	-0.039(-0.093,0.014)	0.03289	705	1.78
C3-DC(C4-OH)	**PCaa32:1**	-0.098(-0.245,0.048)	0.03966	678.7	1.52
C3-DC(C4-OH)	**PCaa40:6**	-0.09(-0.217,0.036)	0.03498	662.9	1.57
C5-DC(C6-OH)	**PCaa42:0**	-1.434(-3.646,0.779)	0.04858	629.4	1.42
C5-DC(C6-OH)	**PCaa42:5**	-0.166(-0.414,0.082)	0.04867	597.6	1.67
C3-DC(C4-OH)	**PCae32:2**	-2.34(-5.403,0.723)	0.02472	592	1.55
C5-DC(C6-OH)	**PCae36:0**	-0.353(-0.848,0.141)	0.03363	577	2.15
C5-DC(C6-OH)	**PCae40:2**	-0.688(-1.594,0.218)	0.0287	569.2	2.51
C3-DC(C4-OH)	**PCaa40:4**	-0.46(-1.106,0.186)	0.03776	539.8	1.63
C3-DC(C4-OH)	**PCae34:2**	-0.299(-0.719,0.122)	0.03454	533.2	1.73
C3-DC(C4-OH)	**PCaa38:5**	-0.006(-0.016,0.003)	0.03937	528.6	1.54
C3-DC(C4-OH)	**PCae40:5**	-0.274(-0.643,0.094)	0.03403	511.9	1.91
C3-DC(C4-OH)	**PCae36:4**	-0.057(-0.137,0.023)	0.03703	483.9	1.61
C3-DC(C4-OH)	**PCae40:1**	-0.226(-0.499,0.047)	0.0241	445.9	1.93
C5-DC(C6-OH)	**PCaa36:2**	-0.005(-0.012,0.002)	0.04477	430.9	1.61
C5-DC(C6-OH)	**PCae40:4**	-0.089(-0.208,0.03)	0.03866	415	1.88
C3-DC(C4-OH)	**PCae42:3**	-0.727(-1.707,0.253)	0.04021	327.2	1.52
C5-DC(C6-OH)	**PCaa42:6**	-0.06(-0.141,0.021)	0.02807	299.3	1.73
C5-DC(C6-OH)	**PCae32:2**	-0.227(-0.557,0.103)	0.03776	263	1.47
C3-DC(C4-OH)	**PCaa40:2**	-5.553(-13.317,2.21)	0.03541	256.4	1.66
C3-DC(C4-OH)	**PCae38:4**	-0.281(-0.697,0.136)	0.04857	188.7	1.48
C3-DC(C4-OH)	**PCae42:1**	-1.397(-3.214,0.419)	0.03271	180.4	1.82
C5-DC(C6-OH)	**PCae40:1**	-0.022(-0.052,0.008)	0.03985	170.3	1.94
C3-DC(C4-OH)	**SM24:1**	-1.096(-2.811,0.619)	0.04995	655.3	1.46
C3-DC(C4-OH)	**SM16:1**	-0.512(-1.245,0.222)	0.03352	485.2	1.44
C3-DC(C4-OH)	**SM24:0**	-2.213(-5.331,0.905)	0.03565	295	1.61
C3-DC(C4-OH)	**lysoPC26:1**	-0.183(-0.393,0.026)	0.01314	10944.4	1.62
C3-DC(C4-OH)	**lysoPC28:1**	-3.227(-6.906,0.451)	0.01595	8112	2.04
C5-DC(C6-OH)	**lysoPC26:1**	-0.018(-0.042,0.005)	0.01706	4363.2	1.59
C3-DC(C4-OH)	**lysoPC28:0**	-2.304(-4.698,0.09)	0.01647	2951.4	2.01
C3-DC(C4-OH)	**lysoPC24:0**	-2.743(-5.969,0.483)	0.02043	2756	1.73
C3-DC(C4-OH)	**lysoPC26:0**	-2.341(-5.007,0.324)	0.02296	2678.3	2.39
C3-DC(C4-OH)	**lysoPC17:0**	-5.618(-13.768,2.532)	0.0259	2160.6	1.48
C3-DC(C4-OH)	**lysoPC18:2**	-0.218(-0.51,0.075)	0.01595	2151.7	1.31

C5-DC(C6-OH)	**lysoPC28:0**	-0.23(-0.508,0.048)	0.01725	2064.8	2.1
C5-DC(C6-OH)	**lysoPC28:1**	-0.331(-0.81,0.148)	0.02776	1906.3	2.06
C3-DC(C4-OH)	**lysoPC14:0**	-0.354(-0.774,0.065)	0.0156	1396.1	1.59
C3-DC(C4-OH)	**lysoPC20:4**	-0.191(-0.455,0.072)	0.02501	1235.6	1.39
C3-DC(C4-OH)	**lysoPC20:3**	-0.914(-2.267,0.439)	0.03195	1170.9	1.34
C5-DC(C6-OH)	**lysoPC18:2**	-0.022(-0.054,0.01)	0.02135	913.7	1.27
C5-DC(C6-OH)	**lysoPC26:0**	-0.237(-0.565,0.091)	0.04477	836.3	2.32
C5-DC(C6-OH)	**lysoPC24:0**	-0.277(-0.658,0.105)	0.03802	810.6	1.77
C3-DC(C4-OH)	**lysoPC16:1**	-2.275(-5.543,0.992)	0.0287	692.4	1.47
C5-DC(C6-OH)	**lysoPC14:0**	-0.035(-0.084,0.014)	0.02432	411.1	1.58
C5-DC(C6-OH)	**lysoPC20:4**	-0.019(-0.049,0.011)	0.0489	397.9	1.34
C3-DC(C4-OH)	**lysoPC18:0**	-0.08(-0.199,0.038)	0.03194	308.6	1.31
C3-DC(C4-OH)	**lysoPC18:1**	-0.103(-0.251,0.044)	0.02645	230.9	1.42

SGLT2-Inhibitor adrenal gland 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
C3-DC(C4-OH)		-0.141(-0.314,0.032)	0.02325		3.25
C3-DC(C4-OH)	**Sum of hexoses**	0(-0.001,0)	0.00546	39031.1	7.7
C2	**Sum of hexoses**	-0.006(-0.014,0.001)	0.04981	147.4	3.31
Sum of hexoses	**PCaa34:3**	54.91(-0.211,110.03)	0.03407	274.7	-2.33
Sum of hexoses	**PCaa36:2**	2.279(-0.023,4.581)	0.02896	416.3	-2.24
C3-DC(C4-OH)	**C5-OH(C3-DC-M)**	-1.904(-3.747,-0.061)	0.01843	16077.5	2.73
C3-DC(C4-OH)	**C7-DC**	-6.996(-14.445,0.453)	0.02325	13342.2	3.52
C3-DC(C4-OH)	**C5:1**	-5.062(-10.251,0.127)	0.02883	5196.5	3.04
C3-DC(C4-OH)	**C3-OH**	-2.194(-4.602,0.214)	0.03483	2867.8	3.02
C3-DC(C4-OH)	**C5-DC(C6-OH)**	-2.865(-5.07,-0.66)	0.01843	2458.8	2.44
C3-DC(C4-OH)	**C4:1**	-9.983(-19.845,-0.121)	0.03271	1631.9	2.74
C14	**C16**	0.073(-0.01,0.155)	0.04703	1052.8	-2.24
C3-DC(C4-OH)	**Creatinine**	-0.047(-0.103,0.009)	0.02635	10634.8	2.53
C3-DC(C4-OH)	**Taurine**	0(-0.001,0)	0.03056	2927.7	2.39
C3-DC(C4-OH)	**Putrescine**	-0.289(-0.639,0.06)	0.01965	1699.4	3.94
Ile	**Thr**	-0.502(-0.746,-0.258)	0.00073	439604.3	4.59
His	**Ile**	0.347(0.095,0.6)	0.00546	25257.7	-2.48
Ile	**Putrescine**	-17.34(-31.65,-3.023)	0.00546	18801.8	4.99
Leu	**Putrescine**	-27.34(-53.05,-1.634)	0.01017	16208.4	4.74
Histamine	**Ile**	0.085(0.007,0.163)	0.00921	6941.1	-1.77
Putrescine	**Val**	0.038(0.001,0.075)	0.01777	3187.4	-1.77
Ile	**Phe**	-0.793(-1.406,-0.18)	0.01299	2711.2	3.01
Thr	**Val**	0.475(-0.023,0.973)	0.01965	2227.1	-1.57
Leu	**Thr**	-0.707(-1.352,-0.062)	0.01965	2078	2.66
Gly	**Ile**	3.956(0.16,7.752)	0.01537	1300.2	-1.76
Gln	**Putrescine**	-166.88(-347.34,13.58)	0.02325	1079.7	3.14
Ile	**Lys**	-0.562(-1.183,0.059)	0.03407	103.7	1.71
Ile	**Tyr**	-1.027(-2.095,0.041)	0.02926	151.8	2.06
Ile	**Trp**	-1.617(-3.345,0.11)	0.02896	159.7	2.5

Cit	**Putrescine**	-3.189(-6.891,0.513)	0.04729	215.9	2.85
Ile	**Met**	-1.574(-3.159,0.011)	0.02462	258.2	2.55
ADMA	**Putrescine**	-0.279(-0.609,0.052)	0.03703	318	3.45
PCaa24:0	**PCaa40:6**	0.003(0,0.006)	0.03056	1373.2	-1.95
PCaa24:0	**PCae34:2**	0.013(-0.002,0.029)	0.04557	1100.3	-1.57
PCaa24:0	**PCae36:3**	0.051(-0.005,0.107)	0.04358	889.4	-1.68
PCaa24:0	**PCaa40:5**	0.001(0,0.003)	0.03843	684	-1.59
C3-DC(C4-OH)	**PCaa36:4**	0(-0.001,0)	0.01299	88897.3	3.06
C3-DC(C4-OH)	**PCaa28:1**	-0.803(-1.494,-0.112)	0.01299	86774.5	2.74
C3-DC(C4-OH)	**PCaa24:0**	-1.09(-2.087,-0.093)	0.01017	76140.1	3.71
C3-DC(C4-OH)	**PCaa40:1**	-0.457(-0.859,-0.055)	0.01299	73609.7	3.42
C3-DC(C4-OH)	**PCae30:0**	-1.147(-2.245,-0.05)	0.01549	63600	3.89
C3-DC(C4-OH)	**PCae30:2**	-3.832(-7.918,0.253)	0.01777	35722.8	3.13
C3-DC(C4-OH)	**PCaa36:5**	-0.021(-0.042,-0.001)	0.01645	34604.4	2.58
C3-DC(C4-OH)	**PCae44:4**	-1.145(-2.266,-0.025)	0.01869	26258.8	3.16
C3-DC(C4-OH)	**PCae42:5**	-0.147(-0.281,-0.013)	0.01843	23007.8	2.86
C3-DC(C4-OH)	**PCae38:4**	-0.022(-0.045,0.002)	0.02325	13705.8	2.32
C3-DC(C4-OH)	**PCae36:0**	-0.456(-1,0.089)	0.02325	12507.6	2.77
C3-DC(C4-OH)	**PCaa38:0**	-0.181(-0.383,0.022)	0.02325	11281.2	2.17
C3-DC(C4-OH)	**PCaa26:0**	-0.193(-0.377,-0.01)	0.02555	11177.8	3.44
C3-DC(C4-OH)	**PCaa42:0**	-1.576(-3.477,0.326)	0.02643	11032.8	2.85
C3-DC(C4-OH)	**PCae34:3**	-0.142(-0.3,0.016)	0.02045	10593.5	2.54
C3-DC(C4-OH)	**PCae42:0**	-0.082(-0.156,-0.008)	0.01777	10234.4	2.7
C3-DC(C4-OH)	**PCae44:5**	-1.457(-3.135,0.222)	0.02849	9573.6	2.81
C3-DC(C4-OH)	**PCaa38:4**	0(-0.001,0)	0.0217	9551	2.46
C3-DC(C4-OH)	**PCae38:0**	-0.105(-0.213,0.004)	0.02083	8733.9	3.17
C3-DC(C4-OH)	**PCae36:4**	-0.05(-0.114,0.013)	0.02325	7989.5	2.4
C3-DC(C4-OH)	**PCae38:6**	-0.021(-0.046,0.004)	0.02462	7464.4	2.25
C3-DC(C4-OH)	**PCae36:5**	-0.004(-0.008,0.001)	0.02643	6802.5	2.34
C3-DC(C4-OH)	**PCaa30:0**	-0.076(-0.187,0.035)	0.02972	5002.9	2.79
C3-DC(C4-OH)	**PCaa36:6**	-0.471(-0.986,0.045)	0.02585	4775.3	2.35
C3-DC(C4-OH)	**PCae40:4**	-0.046(-0.102,0.009)	0.03271	4627.9	2.2
C3-DC(C4-OH)	**PCae38:5**	-0.016(-0.036,0.004)	0.02896	4321.3	2.11
C3-DC(C4-OH)	**PCaa32:3**	-0.887(-2.106,0.332)	0.02896	3892.5	3.17
C3-DC(C4-OH)	**PCaa34:1**	-0.001(-0.001,0)	0.02896	3891.9	2.21
C3-DC(C4-OH)	**PCae36:1**	-0.054(-0.123,0.015)	0.02896	3781.6	2.42
C3-DC(C4-OH)	**PCaa38:5**	-0.001(-0.002,0)	0.02896	3597.9	2.39
C3-DC(C4-OH)	**PCaa34:4**	-0.208(-0.454,0.039)	0.02938	3524.4	2.1
C3-DC(C4-OH)	**PCae38:2**	-0.206(-0.478,0.067)	0.03659	3220.3	2
C3-DC(C4-OH)	**PCaa42:6**	-0.094(-0.196,0.008)	0.02635	3216.2	2.29
C3-DC(C4-OH)	**PCae36:2**	-0.066(-0.153,0.021)	0.03003	2938.8	2.28
C3-DC(C4-OH)	**PCaa34:2**	-0.001(-0.002,0)	0.03113	2757.7	2.17
C3-DC(C4-OH)	**PCae40:6**	-0.113(-0.259,0.033)	0.03056	2648	2.2
C3-DC(C4-OH)	**PCae40:5**	-0.075(-0.164,0.015)	0.03407	2461.5	2.05
C3-DC(C4-OH)	**PCaa40:2**	-0.253(-0.608,0.103)	0.03407	2443.1	2.46
C3-DC(C4-OH)	**PCaa36:0**	-0.132(-0.285,0.022)	0.04908	2404.2	2.19
C3-DC(C4-OH)	**PCae34:2**	-0.044(-0.102,0.014)	0.03003	2367.3	2.22

C3-DC(C4-OH)	**PCae40:1**	-0.065(-0.149,0.018)	0.03531	2067.6	2.94
C3-DC(C4-OH)	**PCaa36:1**	-0.002(-0.006,0.001)	0.0352	2057.9	2.34
C3-DC(C4-OH)	**PCae36:3**	-0.141(-0.319,0.037)	0.02896	1976.5	1.89
C3-DC(C4-OH)	**PCaa42:1**	-2.119(-5.181,0.943)	0.02986	1860.3	3.46
C3-DC(C4-OH)	**PCae40:3**	-0.306(-0.713,0.101)	0.04703	1817	1.79
C3-DC(C4-OH)	**PCae40:2**	-0.438(-0.988,0.112)	0.03483	1797	2.32
C3-DC(C4-OH)	**PCae42:3**	-0.341(-0.752,0.071)	0.04064	1755.7	2.12
C3-DC(C4-OH)	**PCaa32:1**	-0.014(-0.033,0.006)	0.04144	1618.8	1.77
C3-DC(C4-OH)	**PCaa36:2**	-0.001(-0.002,0)	0.0337	1596.6	2.24
C3-DC(C4-OH)	**PCaa34:3**	-0.018(-0.041,0.005)	0.03271	1446.5	1.95
C3-DC(C4-OH)	**PCaa40:4**	-0.004(-0.01,0.001)	0.04729	774.3	2.12
C3-DC(C4-OH)	**PCaa40:5**	-0.004(-0.009,0.001)	0.04539	546.1	2.17
C3-DC(C4-OH)	**PCaa40:6**	-0.007(-0.016,0.002)	0.04715	648.9	2.07
C3-DC(C4-OH)	**SM(OH)22:2**	-0.083(-0.159,-0.008)	0.01299	34980.6	3.07
C3-DC(C4-OH)	**SM(OH)24:1**	-0.809(-1.784,0.166)	0.01777	27166.6	5.06
C3-DC(C4-OH)	**SM16:1**	-0.095(-0.194,0.004)	0.01299	20600.5	2.8
C3-DC(C4-OH)	**SM16:0**	-0.004(-0.008,0)	0.01777	18792.7	2.89
C3-DC(C4-OH)	**SM24:1**	-0.009(-0.019,0.001)	0.01843	12782.6	3.01
C3-DC(C4-OH)	**SM(OH)22:1**	-0.06(-0.143,0.024)	0.02635	10981	4.06
C3-DC(C4-OH)	**SM24:0**	-0.024(-0.06,0.013)	0.02896	7990.7	4.04
C3-DC(C4-OH)	**SM(OH)16:1**	-0.308(-0.674,0.057)	0.02325	3625.4	2.93
C3-DC(C4-OH)	**SM(OH)14:1**	-0.109(-0.215,-0.004)	0.01965	2313.2	2.55
C3-DC(C4-OH)	**SM18:1**	-0.125(-0.284,0.034)	0.02742	1721.7	2.74
C3-DC(C4-OH)	**lysoPC24:0**	-0.827(-1.61,-0.044)	0.01299	62967.3	4.43
C3-DC(C4-OH)	**lysoPC28:1**	-0.795(-1.511,-0.079)	0.01305	62447.1	3.26
C3-DC(C4-OH)	**lysoPC26:0**	-0.822(-1.768,0.123)	0.01965	27234.3	4.03
C3-DC(C4-OH)	**lysoPC28:0**	-0.675(-1.316,-0.034)	0.02325	9828.8	3.02
C3-DC(C4-OH)	**lysoPC26:1**	-0.05(-0.1,0)	0.03056	6479.1	3.36
C3-DC(C4-OH)	**lysoPC14:0**	-0.115(-0.228,-0.002)	0.02896	6358.5	3.26
C3-DC(C4-OH)	**lysoPC16:1**	-0.594(-1.337,0.149)	0.03187	4250.9	2.46
C3-DC(C4-OH)	**lysoPC18:0**	-0.009(-0.02,0.003)	0.04695	1734.3	2.15

SGLT2-Inhibitor lung 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		1502.63(671.72,2333)	0.00025		-3.65
Ile		-13.88(-28.662,0.902)	0.03286		2.3
Sum of hexoses	**Ile**	67.685(23.97,111.4)	0.00025	224.9	-2.48
C3-DC(C4-OH)	**C4:1**	-45.355(-95.471,4.761)	0.02277	1964.4	2.41
C18:1	**Pro**	-0.01(-0.022,0.001)	0.0339	229	1.96
Arg	**Met**	-0.381(-0.788,0.025)	0.01562	229.9	2.44
C3-DC(C4-OH)	**lysoPC20:4**	-0.159(-0.302,-0.016)	0.00205	10020.9	3.83
C3-DC(C4-OH)	**lysoPC18:1**	-0.09(-0.167,-0.012)	0.00602	2076.9	2.62
C18:1	**lysoPC18:2**	-0.027(-0.059,0.005)	0.02591	252.1	2.4
C3-DC(C4-OH)	**lysoPC18:2**	-0.04(-0.089,0.008)	0.02856	227.3	1.81
C3-DC(C4-OH)	**lysoPC20:3**	-0.65(-1.377,0.077)	0.02474	184.4	1.97

C5-DC(C6-OH)	**lysoPC17:0**	-0.041(-0.088,0.006)	0.04411	133.8	1.77
lysoPC17:0	**Spermidine**	0.019(-0.005,0.044)	0.01972	424.4	-1.31

SGLT2-Inhibitor cerebellum 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Ile		-2.756(-4.718,-0.794)	0.00072		5.2
Arg		-3.779(-7.409,-0.149)	0.00672		4.3
Arg	**Phe**	-0.778(-1.236,-0.32)	0.00038	910.3	5.57
Arg	**PCaa36:6**	-19.36(-32.71,-6.015)	0.00038	377.5	7.08

SGLT2-Inhibitor plasma 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		14063(3915,24210)	0.00003		-2.48
Putrescine		-0.533(-0.902,-0.163)	0.00003		6.05
Spermidine		-4.926(-10.081,0.229)	0.00003		17.05
C18:1(OH)		-0.006(-0.011,-0.001)	0.0003		3.02
Pro		42.35(-4.559,89.259)	0.00062		-1.47
C3-DC(C4-OH)		-0.098(-0.189,-0.007)	0.0015		3.47
Thr		64.86(-9.258,138.978)	0.00203		-1.44
Ile		-91.82(-189.105,5.465)	0.00283		2.01
Met		13.62(-4,31.24)	0.00331		-1.39
Tyr		18.31(-4.236,40.856)	0.00392		-1.42
C16		-0.082(-0.174,0.01)	0.00434		2.36
Ser		31.56(-8.15,71.27)	0.00496		-1.85
Asn		15.04(-4.324,34.404)	0.00607		-1.38
C10		0.061(-0.024,0.147)	0.00608		-1.24
C18:1		-0.098(-0.219,0.024)	0.00764		1.96
C0		4.372(-1.783,10.527)	0.01001		-1.38
C4:1		0.027(-0.011,0.064)	0.01117		-1.4
Gly		35.08(-22.925,93.085)	0.01325		-1.3
C16:1		-0.015(-0.037,0.007)	0.01417		1.64
lysoPC17:0		0.378(-0.234,0.99)	0.01606		-1.01
C16:1(OH)		-0.003(-0.007,0.002)	0.02607		2.06
C14		-0.016(-0.043,0.011)	0.02991		1.45
Ala		148.9(-114.93,412.73)	0.03006		-0.92
Spermine		-1.953(-7.559,3.653)	0.03246		17.01
Leu		-143.29(-433.5,146.97)	0.03375		1.18
Glu		-32.64(-104.94,39.66)	0.03833		3.38
His		15.59(-14.306,45.486)	0.04236		-1.01
Cit		15.57(-16.449,47.589)	0.04496		-0.84
C5-DC(C6-OH)		-0.004(-0.012,0.004)	0.04562		1.57
C18:1(OH)	**Sum of hexoses**	0(0,0)	0.00003	153.5	9.13
C3-DC(C4-OH)	**C6(C4:1-DC)**	-1.774(-2.99,-0.558)	0.00006	1014026.6	5.39

C3-DC(C4-OH)	**C5-OH(C3-DC-M)**	-1.553(-3.054,-0.053)	0.00057	30505.8	5.1
C3-DC(C4-OH)	**C8**	-0.994(-1.897,-0.091)	0.00106	12100.2	2.97
C3-DC(C4-OH)	**C6:1**	-5.584(-10.152,-1.016)	0.00028	11281.6	3.04
C10	**C16**	0.605(0.245,0.965)	0.00003	10058.3	-3.13
C3-DC(C4-OH)	**C4:1**	-2.242(-3.765,-0.719)	0.00006	4214.1	4.6
C3-DC(C4-OH)	**C5:1**	-2.483(-5.054,0.088)	0.00275	2705.6	2.26
C10	**C18:1**	0.734(0.237,1.232)	0.00003	2114.6	-2.66
C5-DC(C6-OH)	**C5-OH(C3-DC-M)**	-0.059(-0.136,0.018)	0.00972	472.3	1.58
C10	**C16:1**	2.21(0.419,4.002)	0.00008	427.4	-2.18
C3-DC(C4-OH)	**C5**	-0.608(-1.263,0.047)	0.00219	375.3	2.34
C3-DC(C4-OH)	**C9**	-4.568(-10.343,1.207)	0.01044	286.9	1.65
C4:1	**C5:1**	0.768(0.106,1.431)	0.00024	253.3	-2.07
C18:1	**C4:1**	-2.747(-5.156,-0.337)	0.00016	253.2	4.27
C3-DC(C4-OH)	**C5-M-DC**	-4.401(-9.69,0.888)	0.00886	239.8	2.61
C10	**C3-DC(C4-OH)**	1.424(0.299,2.549)	0.00006	164.4	-2.24
C16	**C4:1**	-2.521(-4.503,-0.539)	0.00013	155.9	4.25
C10	**C18**	1.088(0.277,1.898)	0.0002	120.4	-2.9
C10	**C14**	1.989(0.24,3.737)	0.00022	117.1	-2.01
C10	**C16(OH)**	9.243(1.569,16.916)	0.00024	104.5	-2.32
C10	**C14:1**	1.281(0.251,2.311)	0.00024	103.4	-2.66
C3-DC(C4-OH)	**Trp**	-0.001(-0.003,0)	0.00089	3129.1	3.81
C3-DC(C4-OH)	**Creatinine**	-0.011(-0.023,0)	0.00292	2767.2	3.15
C5-DC(C6-OH)	**Taurine**	0(0,0)	0.00038	1877.9	7.3
C3-DC(C4-OH)	**Phe**	-0.001(-0.003,0)	0.0012	1246.1	3.84
C3-DC(C4-OH)	**Lys**	-0.001(-0.001,0)	0.00118	919.4	3.63
C3-DC(C4-OH)	**His**	-0.002(-0.003,0)	0.00061	562.5	3.44
C5-DC(C6-OH)	**Gly**	0(0,0)	0.00016	528	4.19
Ala	**C5-DC(C6-OH)**	15881(1178,30584)	0.00048	451.3	-1.94
C3-DC(C4-OH)	**Taurine**	0(-0.001,0)	0.00119	360.3	3.99
C0	**Spermidine**	9.49(2.81,16.169)	0.00003	348.5	-2.26
C5-DC(C6-OH)	**Lys**	0(0,0)	0.00245	311.4	2.96
C3-DC(C4-OH)	**Cit**	-0.002(-0.004,0)	0.00103	288	3.12
C3-DC(C4-OH)	**Gln**	0(0,0)	0.00639	222.7	2.09
C3-DC(C4-OH)	**Kynurenine**	-0.112(-0.29,0.065)	0.01642	207.4	3.08
C3-DC(C4-OH)	**MetSO**	-0.127(-0.272,0.017)	0.00352	203.1	3.11
C3-DC(C4-OH)	**Carnosine**	-0.374(-0.883,0.135)	0.01507	200.5	2.34
C5:1-DC	**Phe**	0(0,0)	0.00359	120.2	2.61
C10	**Ile**	0.001(0,0.002)	0.00009	111	-1.89
Glu	**Gly**	-0.429(-0.762,-0.096)	0.00006	6305.2	5.37
Glu	**Taurine**	-0.15(-0.292,-0.008)	0.00013	3946.7	7.92
Gln	**Met**	-10.81(-17.82,-3.804)	0.00003	1040.6	4.5
Glu	**Lys**	-0.18(-0.369,0.009)	0.00038	888.5	5.46
Glu	**Met**	-1.864(-3.472,-0.257)	0.00006	685.7	7.79
Ile	**Thr**	-1.649(-2.607,-0.692)	0.00003	664.6	4.86
Glu	**Ser**	-0.519(-1.018,-0.019)	0.00008	470	8.06
Ile	**Met**	-5.665(-9.058,-2.273)	0.00006	415.7	4
Gly	**Spermidine**	99.61(47.76,151.46)	0.00003	372	-3.14

Gln	**Ser**	-2.735(-4.728,-0.743)	0.00008	338.7	3.55
Ala	**Glu**	3.556(0.308,6.804)	0.00068	286	-1.94
Ile	**Phe**	-1.284(-2.188,-0.38)	0.00008	187.7	3.93
Gln	**His**	-2.613(-5.086,-0.14)	0.00131	181.9	1.99
Ile	**Pro**	-3.21(-4.834,-1.586)	0.00003	168.6	4.62
Gln	**Lys**	-0.769(-1.71,0.171)	0.00377	167.2	2.03
Ile	**Tyr**	-2.402(-3.935,-0.869)	0.00008	157.1	3.17
Thr	**Val**	0.242(0.045,0.44)	0.00006	151.9	-1.99
Glu	**His**	-0.603(-1.324,0.118)	0.00141	138.8	4.72
Gln	**Phe**	-1.478(-3.405,0.448)	0.0061	114.9	1.75
Pro	**Spermidine**	71.27(23.92,118.61)	0.00003	106.5	-2.41
His	**Ile**	0.265(0.014,0.516)	0.00009	103.9	-1.74
His	**Val**	0.091(-0.007,0.189)	0.00197	96.2	-1.55
PCaa36:5	**PCae40:5**	0.943(0.432,1.454)	0.00003	2892824.5	-4.06
PCaa38:0	**PCae38:3**	-0.267(-0.412,-0.122)	0.00003	833376.9	3.86
PCaa36:5	**PCae38:5**	0.376(0.113,0.639)	0.00006	91697.1	-3
PCaa36:5	**PCae34:1**	0.747(0.231,1.262)	0.00008	42490.4	-3.31
PCaa36:5	**PCae36:4**	0.324(0.09,0.557)	0.00008	41538	-3.4
PCaa32:0	**PCaa34:4**	-3.303(-5.366,-1.24)	0.00006	27386.7	2.79
PCaa34:4	**PCae38:5**	0.065(0.017,0.113)	0.00006	18362.9	-2.69
PCaa34:4	**PCae34:1**	0.13(0.024,0.236)	0.00013	6062.8	-2.29
PCaa34:4	**PCae36:4**	0.057(0.012,0.102)	0.00013	5935.9	-2.72
PCaa36:5	**PCae38:6**	0.578(0.049,1.107)	0.00038	5522.7	-2.04
PCaa34:3	**PCae38:5**	0.79(0.03,1.55)	0.0005	5482.1	-1.9
PCaa34:3	**PCae40:5**	1.812(0.127,3.497)	0.0007	4702	-3.18
PCae38:4	**PCae40:5**	0.499(-0.005,1.004)	0.00151	3752.4	-3.25
PCaa32:2	**PCae38:5**	0.052(0.003,0.101)	0.00064	3493.6	-1.87
PCaa34:3	**PCae36:4**	0.662(-0.001,1.324)	0.00088	3408	-1.92
PCaa34:3	**PCae34:1**	1.551(0.186,2.917)	0.00046	3131.8	-2.58
PCaa38:0	**PCae42:5**	-0.502(-0.92,-0.083)	0.00028	3096.4	2.73
PCaa34:4	**PCaa38:0**	0.182(0.018,0.347)	0.00022	2817.3	-1.93
PCaa38:0	**PCae44:4**	-4.349(-8.266,-0.431)	0.0004	1787	3.27
PCaa34:4	**PCae38:6**	0.101(0.014,0.189)	0.00033	1748.2	-2.34
PCaa36:5	**PCaa38:0**	1.05(0.118,1.983)	0.00042	1735.2	-2.49
PCaa34:4	**PCae40:5**	0.174(0.011,0.337)	0.00037	1537.6	-1.87
PCaa32:2	**PCae34:1**	0.104(0.006,0.203)	0.00088	1265.8	-1.86
PCae40:4	**PCae40:5**	0.195(-0.034,0.425)	0.00333	1167.4	-1.65
PCae42:3	**PCae44:3**	2.551(0.052,5.049)	0.00094	1128.1	-2.09
PCae38:4	**PCae38:5**	0.241(-0.003,0.485)	0.00155	1077.2	-2.21
PCaa32:3	**PCaa34:4**	-0.04(-0.076,-0.004)	0.00048	1016.9	2.93
PCaa42:4	**PCae42:3**	-0.088(-0.186,0.011)	0.00196	1003.3	2.69
PCaa36:5	**PCaa42:4**	5.195(0.075,10.315)	0.00124	993.9	-2.08
PCaa38:0	**PCae44:6**	-2.723(-5.279,-0.167)	0.0007	857.2	2.66
PCaa32:2	**PCaa38:0**	0.15(0.001,0.299)	0.00071	841.2	-1.7
PCaa34:3	**PCae38:6**	1.192(-0.241,2.625)	0.00228	827	-1.38
PCaa42:4	**PCae40:4**	-0.021(-0.045,0.003)	0.00354	793.5	1.81
PCaa42:5	**PCae42:3**	-0.087(-0.186,0.011)	0.00238	752.7	2.21

PCaa36:5	**PCaa42:5**	4.707(-0.166,9.58)	0.00151	743	-1.79
PCaa32:2	**PCae36:4**	0.044(-0.003,0.091)	0.00193	681.9	-1.81
PCaa36:6	**PCae38:5**	0.027(-0.001,0.055)	0.00222	639.3	-3.3
PCaa36:4	**PCae38:6**	16.344(-3.245,35.932)	0.00383	589.7	-1.93
PCaa42:1	**PCae44:4**	-0.353(-0.72,0.014)	0.00156	549.7	2.48
PCae36:4	**PCae38:4**	-0.128(-0.286,0.03)	0.00427	514.1	2.62
PCae38:4	**PCae38:6**	0.344(-0.077,0.764)	0.00421	506.7	-1.55
PCae42:1	**PCae42:3**	-0.189(-0.407,0.029)	0.00333	463	2.6
PCaa36:5	**PCae40:6**	0.438(-0.035,0.911)	0.00215	442.6	-2.1
PCaa42:1	**PCae42:3**	-0.064(-0.133,0.005)	0.00207	366	3.23
PCaa34:3	**PCaa42:5**	9.223(-1.491,19.936)	0.00418	338.4	-1.79
PCaa38:0	**PCae36:1**	-0.195(-0.379,-0.01)	0.00136	324.8	1.88
PCaa40:4	**PCae40:4**	-0.262(-0.631,0.106)	0.00796	318.8	2.09
PCaa32:0	**PCaa36:5**	-0.416(-0.825,-0.007)	0.00152	316	2.01
PCaa36:6	**PCae38:6**	0.042(-0.007,0.09)	0.00419	289.2	-2.06
PCaa32:0	**PCae36:2**	-0.29(-0.62,0.04)	0.00164	279	5.27
PCaa32:0	**PCaa32:2**	-2.555(-5.17,0.059)	0.00174	255.8	1.62
PCaa36:6	**PCae36:4**	0.023(-0.004,0.05)	0.00504	223.1	-3.38
PCaa42:1	**PCae42:2**	-0.055(-0.12,0.009)	0.00302	210.6	2.05
PCaa36:5	**PCae44:3**	19.238(-4.262,42.739)	0.00305	199.5	-1.33
PCae34:1	**PCae38:4**	-0.085(-0.186,0.016)	0.00309	194.6	2.81
PCaa32:2	**PCae40:5**	0.126(-0.032,0.285)	0.00463	190.9	-1.48
PCae34:1	**PCae40:4**	-0.228(-0.492,0.035)	0.00331	175.1	2.08
PCaa38:4	**PCae40:5**	26.49(-10.69,63.68)	0.01147	171.2	-2.94
PCaa38:4	**PCae38:6**	18.109(-6.731,42.949)	0.00874	170.5	-1.58
PCaa34:3	**PCaa42:4**	10.189(-2.672,23.05)	0.0067	170.2	-2.09
PCaa40:4	**PCae42:3**	-1.181(-2.724,0.361)	0.00665	167.4	2.15
PCaa38:0	**PCae40:4**	-0.225(-0.474,0.024)	0.00219	159.5	2.59
PCaa34:3	**PCaa38:0**	2.183(-0.248,4.613)	0.0022	158	-2.49
PCaa34:3	**PCae40:6**	0.791(-0.246,1.827)	0.00704	157.7	-1.55
PCaa42:4	**PCae44:4**	-0.425(-1.026,0.177)	0.00954	152.6	2.01
PCaa34:4	**PCae44:5**	1.928(-0.183,4.039)	0.0018	149	-1.82
PCaa36:3	**PCaa38:0**	14.067(-1.955,30.09)	0.0023	146.9	-1.48
PCaa36:4	**PCae38:5**	11.317(-2.856,25.49)	0.00608	146.6	-2.2
PCaa38:0	**PCae36:2**	-0.069(-0.14,0.002)	0.00231	146	1.89
PCaa42:0	**PCae44:4**	-0.284(-0.69,0.121)	0.00991	144.2	1.86
PCaa34:3	**PCae44:3**	40.681(-9.638,91.001)	0.00387	139.6	-1.33
PCaa36:6	**PCae40:5**	0.068(-0.022,0.157)	0.00712	133.2	-2.13
PCaa32:1	**PCaa42:4**	0.996(-0.461,2.454)	0.01214	126.1	-1.32
PCaa32:3	**PCaa36:5**	-0.004(-0.01,0.001)	0.00507	125.5	2.37
PCaa32:2	**PCae38:6**	0.078(-0.021,0.177)	0.00617	125.5	-1.51
PCaa36:5	**PCaa40:4**	0.303(-0.045,0.651)	0.0051	124.1	-2.12
PCaa38:4	**PCae38:5**	12.601(-3.099,28.301)	0.00677	123.8	-2.01
PCaa36:5	**PCae40:5**	0.078(-0.021,0.176)	0.00512	123.7	-1.33
PCaa38:5	**PCae40:5**	4.572(-2.268,11.412)	0.0144	123.4	-2.94
PCaa36:6	**PCae34:1**	0.054(-0.01,0.117)	0.00424	120.8	-2.4
PCae40:4	**PCae44:3**	4.971(-1.407,11.35)	0.00444	114.2	-1.29

PCae36:5	**PCae38:5**	0.101(-0.031,0.234)	0.00724	111.4	-1.34
PCaa36:6	**PCaa42:5**	0.349(-0.118,0.816)	0.00801	111	-1.43
PCae38:5	**PCae40:4**	-0.436(-1.009,0.136)	0.00753	104.7	1.84
PCaa36:5	**PCaa42:1**	19.987(-5.826,45.801)	0.00504	99.1	-1.39
PCaa40:1	**PCae44:4**	-0.602(-1.452,0.247)	0.00904	98	1.69
SM(OH)16:1	**SM16:1**	0.011(0.001,0.021)	0.00056	1249.4	-1.88
SM16:1	**SM18:0**	-0.688(-1.401,0.026)	0.00066	1020.5	9.26
SM16:1	**SM18:1**	-1.072(-2.246,0.102)	0.00164	260.3	4.03
PCaa34:4	**SM16:1**	0.038(0.017,0.059)	0.00003	256268.8	-4.4
PCaa32:2	**SM16:1**	0.031(0.009,0.052)	0.00006	39336.1	-2.77
PCaa32:2	**SM(OH)22:2**	0.078(0.009,0.146)	0.0005	4883.1	-3.26
PCaa36:5	**SM16:1**	0.221(0.046,0.396)	0.00028	3331.8	-2.89
PCaa34:3	**SM16:1**	0.468(0.049,0.886)	0.00063	1098.1	-2.2
PCae38:5	**SM(OH)24:1**	-5.383(-11.887,1.12)	0.00337	346.9	2.53
PCaa34:4	**SM(OH)22:2**	0.109(0.001,0.217)	0.00119	280.5	-2.91
PCaa36:5	**SM(OH)22:2**	0.583(-0.079,1.246)	0.0036	208.9	-3.95
PCae38:5	**SM18:0**	-0.382(-0.926,0.163)	0.0056	165.5	3.47
PCae38:6	**SM18:0**	-0.18(-0.44,0.081)	0.00644	154.4	3.31
PCae38:6	**SM(OH)24:1**	-2.534(-5.9,0.832)	0.00747	150.6	1.96
PCae36:4	**SM(OH)24:1**	-4.65(-11.249,1.949)	0.00872	118.3	2.35
lysoPC16:1	**PCae34:1**	0.308(0.101,0.516)	0.00006	69864	-3.26
lysoPC17:0	**PCae38:5**	0.126(0.04,0.213)	0.00003	11301.1	-2.57
lysoPC17:0	**PCae36:3**	0.248(0.076,0.419)	0.00006	8503.4	-2.78
lysoPC17:0	**PCae34:1**	0.251(0.071,0.431)	0.00006	7294.7	-2.43
lysoPC17:0	**PCae34:2**	0.141(0.044,0.239)	0.00006	5808.6	-2.87
lysoPC16:1	**PCaa38:0**	0.442(0.072,0.812)	0.00024	3556.3	-3.02
lysoPC18:0	**PCae36:3**	12.304(1.071,23.536)	0.00061	3425.7	-2.25
lysoPC16:1	**PCae38:5**	0.153(0.007,0.3)	0.00112	1765.9	-2.39
lysoPC18:0	**PCae34:1**	16.957(0.635,33.28)	0.00071	1647.6	-1.86
lysoPC17:0	**PCae38:6**	0.192(0.054,0.33)	0.00009	1430.2	-3.76
lysoPC18:0	**PCae38:5**	8.434(0.24,16.629)	0.00118	1306.5	-1.94
lysoPC17:0	**PCaa32:0**	0.066(0.017,0.114)	0.00015	869	-2.99
lysoPC17:0	**PCae36:4**	0.112(0.021,0.203)	0.00016	719.8	-2.28
lysoPC17:0	**PCaa38:0**	0.344(0.077,0.611)	0.00016	702.5	-2.48
lysoPC18:0	**PCaa38:0**	24.535(0.639,48.43)	0.00083	666.4	-1.82
lysoPC16:0	**PCae38:5**	10.94(-1.195,23.076)	0.0025	534.4	-1.6
lysoPC18:0	**PCaa32:0**	4.34(0.242,8.438)	0.0012	449.4	-3.27
lysoPC16:1	**PCae36:4**	0.128(-0.016,0.271)	0.00382	420.8	-2.07
lysoPC16:0	**PCae38:6**	14.963(-3.979,33.906)	0.00543	229.5	-1.91
lysoPC16:1	**PCaa40:4**	0.113(-0.032,0.258)	0.00685	176.1	-3.79
lysoPC16:0	**PCae34:1**	21.876(-3.644,47.396)	0.00342	167.7	-1.57
lysoPC16:1	**PCae40:5**	0.346(-0.1,0.792)	0.00719	164.4	-2.52
lysoPC16:0	**PCaa32:0**	5.564(-0.06,11.188)	0.0025	151.7	-3.32
lysoPC16:1	**PCaa42:4**	2.014(-0.704,4.731)	0.00817	134.6	-1.79
lysoPC24:0	**PCaa42:1**	1.579(-0.379,3.537)	0.00491	103	-1.69
lysoPC18:0	**PCae36:4**	7.084(-1.789,15.957)	0.0067	100.8	-1.57
lysoPC17:0	**SM16:1**	0.074(0.027,0.121)	0.00003	17449	-2.97

lysoPC16:0	**SM16:1**	6.573(0.128,13.017)	0.00082	743.9	-1.77
lysoPC17:0	**SM24:0**	0.026(0.004,0.048)	0.00026	396.6	-2.24
lysoPC18:0	**SM16:1**	4.914(-0.051,9.879)	0.00185	216.5	-2.38
lysoPC18:0	**SM24:0**	1.43(-0.278,3.138)	0.00411	205.2	-1.52
lysoPC16:0	**lysoPC17:0**	-19.741(-37.932,-1.55)	0.00069	107.2	2.58
C3-DC(C4-OH)	**PCaa34:2**	0(0,0)	0.00056	22830.8	3.1
C3-DC(C4-OH)	**PCaa36:2**	0(0,0)	0.00071	15494.1	3.1
C3-DC(C4-OH)	**PCae36:2**	-0.015(-0.029,-0.001)	0.00048	7585	3.43
C3-DC(C4-OH)	**PCaa34:1**	-0.001(-0.002,0)	0.00134	5745.2	2.75
C3-DC(C4-OH)	**PCae36:3**	-0.07(-0.139,-0.001)	0.00208	4525.5	2.7
C3-DC(C4-OH)	**PCaa36:3**	-0.001(-0.002,0)	0.00207	3675	2.75
C3-DC(C4-OH)	**PCae34:2**	-0.035(-0.072,0.001)	0.0023	3551.4	2.36
C3-DC(C4-OH)	**PCae34:3**	-0.124(-0.258,0.009)	0.00156	2807.2	2.45
C3-DC(C4-OH)	**PCae36:0**	-0.351(-0.705,0.002)	0.00154	2549.7	3.29
C3-DC(C4-OH)	**PCaa32:3**	-0.515(-1.09,0.061)	0.00278	2539.3	2.17
C3-DC(C4-OH)	**PCae36:5**	-0.048(-0.096,-0.001)	0.00207	2293	2.36
C3-DC(C4-OH)	**PCae42:5**	-0.151(-0.312,0.01)	0.0033	2277.8	2.51
C3-DC(C4-OH)	**PCaa30:0**	-0.123(-0.256,0.009)	0.00319	2234.4	2.42
C3-DC(C4-OH)	**PCaa36:1**	-0.002(-0.004,0.001)	0.00269	2087.7	3.3
C3-DC(C4-OH)	**PCaa28:1**	-0.214(-0.443,0.015)	0.00163	1354.3	2.12
C3-DC(C4-OH)	**PCae38:2**	-0.023(-0.053,0.006)	0.00476	1236.3	2.37
C4:1	**PCaa36:0**	0.027(0.004,0.05)	0.00008	1167.1	-2.03
C3-DC(C4-OH)	**PCaa32:2**	-0.129(-0.258,-0.001)	0.00136	1164.4	2.33
C3-DC(C4-OH)	**PCaa36:4**	0(-0.001,0)	0.00307	1047.1	2.68
C3-DC(C4-OH)	**PCaa40:5**	-0.01(-0.022,0.002)	0.00481	1014.9	2.48
C3-DC(C4-OH)	**PCae44:6**	-0.696(-1.52,0.129)	0.00442	949.7	2.61
C3-DC(C4-OH)	**PCae36:1**	-0.046(-0.096,0.005)	0.00315	917.4	2.38
C3-DC(C4-OH)	**PCaa38:4**	0(-0.001,0)	0.0038	895.8	2.55
C3-DC(C4-OH)	**PCae38:3**	-0.069(-0.15,0.013)	0.00481	863.5	2.39
C3-DC(C4-OH)	**PCaa34:4**	-0.147(-0.281,-0.012)	0.00064	688	3.11
C3-DC(C4-OH)	**PCae38:4**	-0.019(-0.042,0.004)	0.00483	682.4	2.24
C3-DC(C4-OH)	**PCaa34:3**	-0.008(-0.016,0.001)	0.00269	656.2	2.36
C3-DC(C4-OH)	**PCaa36:5**	-0.02(-0.041,0.001)	0.00179	570.8	2.69
C3-DC(C4-OH)	**PCae40:6**	-0.045(-0.103,0.013)	0.00887	552.5	1.99
C3-DC(C4-OH)	**PCaa36:6**	-0.274(-0.596,0.049)	0.00279	532.4	2.68
C3-DC(C4-OH)	**PCae44:4**	-0.982(-2.101,0.137)	0.00424	510.9	2.64
C3-DC(C4-OH)	**PCae38:1**	-0.09(-0.224,0.044)	0.00862	500.2	2.61
C3-DC(C4-OH)	**PCae42:4**	-0.384(-0.872,0.103)	0.00789	497.8	2.06
C3-DC(C4-OH)	**PCaa38:5**	-0.002(-0.005,0.001)	0.00626	431.3	2.3
C3-DC(C4-OH)	**PCae40:4**	-0.052(-0.117,0.013)	0.00709	381.3	2.15
C3-DC(C4-OH)	**PCae32:2**	-0.461(-1.096,0.175)	0.01114	378.4	1.82
C5-DC(C6-OH)	**PCaa28:1**	-0.012(-0.028,0.005)	0.00413	345.8	2.4
C3-DC(C4-OH)	**PCaa40:6**	-0.002(-0.004,0.001)	0.01173	341.4	2.13
C3-DC(C4-OH)	**PCae34:0**	-0.446(-1.034,0.142)	0.00985	328.3	2.15
C3-DC(C4-OH)	**PCaa32:1**	-0.047(-0.108,0.014)	0.01114	267.1	1.95
C3-DC(C4-OH)	**PCaa38:6**	-0.001(-0.002,0)	0.01344	255	2.12
C3-DC(C4-OH)	**PCae40:1**	-0.032(-0.076,0.012)	0.00715	254.8	2.33

C3-DC(C4-OH)	PCae44:5	-0.802(-1.897,0.294)	0.01015	253.3	2.01
C3-DC(C4-OH)	PCae38:0	-0.042(-0.106,0.022)	0.01143	244	2
C3-DC(C4-OH)	PCae40:5	-0.066(-0.156,0.023)	0.01083	230.8	1.84
C3-DC(C4-OH)	PCaa38:3	-0.001(-0.003,0)	0.01325	229.9	1.85
C5-DC(C6-OH)	PCae36:1	-0.002(-0.005,0.001)	0.00824	220.4	1.79
C3-DC(C4-OH)	PCaa26:0	-0.114(-0.244,0.016)	0.00715	218.6	2.23
C3-DC(C4-OH)	PCae40:2	-0.138(-0.339,0.063)	0.01377	196.2	1.65
C3-DC(C4-OH)	PCae42:2	-0.187(-0.475,0.1)	0.01801	188.6	1.84
C3-DC(C4-OH)	PCae38:6	-0.033(-0.074,0.008)	0.00847	179.5	1.99
C3-DC(C4-OH)	PCae32:1	-0.179(-0.436,0.077)	0.01641	158.7	1.51
C18:1	PCae36:2	-0.016(-0.027,-0.004)	0.00022	157.1	2.72
C5-DC(C6-OH)	PCae38:3	-0.003(-0.007,0.002)	0.01501	155.8	1.61
C3-DC(C4-OH)	PCae40:3	-0.102(-0.257,0.054)	0.0183	123.4	1.6
C3-DC(C4-OH)	PCae42:3	-0.157(-0.372,0.058)	0.00824	120.2	2.37
C3-DC(C4-OH)	PCae30:2	-0.745(-1.886,0.396)	0.02039	119.8	1.66
C3-DC(C4-OH)	PCae36:4	-0.018(-0.042,0.006)	0.01144	119	1.79
C3-DC(C4-OH)	PCae42:0	-0.138(-0.334,0.057)	0.01481	115.7	2.02
C5-DC(C6-OH)	PCaa30:0	-0.005(-0.015,0.004)	0.02304	115.3	1.87
C10	PCae34:1	0.036(0.008,0.064)	0.00022	114.4	-3.36
C0	PCaa40:3	6.965(0.199,13.73)	0.0004	110.1	-1.67
C5-DC(C6-OH)	PCae36:2	-0.001(-0.002,0)	0.00897	101.9	1.53
C3-DC(C4-OH)	SM18:0	-0.034(-0.066,-0.002)	0.00054	5855.8	2.83
C3-DC(C4-OH)	SM16:0	-0.002(-0.005,0)	0.0016	5832.4	2.03
C3-DC(C4-OH)	SM(OH)14:1	-0.037(-0.077,0.003)	0.00154	4601.5	1.86
C3-DC(C4-OH)	SM(OH)22:1	-0.021(-0.043,0)	0.00155	4136.7	1.88
C3-DC(C4-OH)	SM18:1	-0.072(-0.148,0.005)	0.00214	2453.4	2.42
C3-DC(C4-OH)	SM24:0	-0.006(-0.013,0.001)	0.0034	1785.8	2.21
C3-DC(C4-OH)	SM(OH)24:1	-0.489(-1.025,0.047)	0.00151	1586.8	2.51
C3-DC(C4-OH)	SM(OH)16:1	-0.26(-0.55,0.03)	0.0027	1253.1	1.98
C3-DC(C4-OH)	SM(OH)22:2	-0.045(-0.1,0.01)	0.0049	968.8	1.63
C4:1	SM24:1	0.003(0,0.005)	0.00026	232.4	-2.54
C10	SM16:1	0.011(0.002,0.02)	0.0002	125.6	-2.49
C3-DC(C4-OH)	lysoPC18:2	-0.001(-0.002,0)	0.00052	6229	4.98
C3-DC(C4-OH)	lysoPC14:0	-0.014(-0.026,-0.002)	0.00135	5711.4	3.89
C3-DC(C4-OH)	lysoPC16:0	0(-0.001,0)	0.00074	4382.2	3.44
C3-DC(C4-OH)	lysoPC18:1	-0.004(-0.007,0)	0.00088	2628.6	4.68
C3-DC(C4-OH)	lysoPC18:0	-0.001(-0.001,0)	0.0008	2306.2	3.37
C3-DC(C4-OH)	lysoPC20:4	-0.002(-0.005,0)	0.00319	1436.7	3.42
C3-DC(C4-OH)	lysoPC26:1	-0.05(-0.103,0.004)	0.0049	727	2.65
C3-DC(C4-OH)	lysoPC16:1	-0.038(-0.079,0.003)	0.00362	455.4	2.74
C3-DC(C4-OH)	lysoPC24:0	-0.151(-0.34,0.038)	0.00711	424.4	2.16
C3-DC(C4-OH)	lysoPC17:0	-0.077(-0.135,-0.019)	0.0003	338.2	3.67
C3-DC(C4-OH)	lysoPC20:3	-0.01(-0.023,0.003)	0.01104	317.9	3.35
C16	lysoPC17:0	-0.078(-0.135,-0.021)	0.00009	198.3	3.59
C5-DC(C6-OH)	lysoPC24:0	-0.007(-0.018,0.003)	0.01182	198.1	2.05
C16(OH)	lysoPC17:0	-0.003(-0.005,0)	0.00048	172.4	3.88
C18:1	lysoPC17:0	-0.087(-0.156,-0.019)	0.00022	153.8	3.42

C3-DC(C4-OH)	lysoPC28:1	-0.212(-0.511,0.088)	0.01742	110.9	1.7
Ile	lysoPC17:0	-84.82(-144.9,-24.74)	0.00006	254.6	4.38

Combination treatment

Combination treatment testis 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		287.07(44.37,529.77)	0.00098		-2.54
Cit		2.793(0.393,5.193)	0.00241		-2.3
C3		-0.283(-0.61,0.045)	0.01663		2.76
lysoPC18:2		0.574(-0.063,1.21)	0.02095		-1.83
Arg	Ile	0.262(-0.005,0.529)	0.00446	159	-1.75
His	Putrescine	-4.452(-10.345,1.441)	0.03566	144.1	2.57

Combination treatment adrenal gland 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
C3-DC(C4-OH)	Sum of hexoses	0(-0.001,0)	0.00175	48939	8.89
C3-DC(C4-OH)	SM24:0	-0.023(-0.056,0.009)	0.12621	4612.8	3.98
C3-DC(C4-OH)	SM(OH)22:1	-0.062(-0.163,0.04)	0.12621	3233	4.18
C3-DC(C4-OH)	C5	-1.73(-3.935,0.474)	0.12621	2891.8	1.97
C3-DC(C4-OH)	lysoPC18:2	-0.033(-0.073,0.006)	0.12621	2269.4	2.16
C3-DC(C4-OH)	PCaa40:1	-0.399(-0.908,0.109)	0.12621	2261.8	2.99
C3-DC(C4-OH)	SM(OH)24:1	-0.835(-2.534,0.865)	0.12621	2236.9	5.22
C3-DC(C4-OH)	lysoPC24:0	-0.695(-1.552,0.163)	0.12621	2092.6	3.72

Combination treatment lung 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		1679(767.4,2592)	0.0004		-4.08
Cit		7.142(2.334,11.95)	0.00056		-3.06
Thr		17.12(-0.841,35.081)	0.01025		-2.05
C3-DC(C4-OH)	Sum of hexoses	-0.002(-0.003,0)	0.00005	176.4	13.96
Sum of hexoses	Serotonin	1591(739.07,2444)	0.00005	103.1	-3.15
C3-DC(C4-OH)	C4:1	-43.406(-89.032,2.22)	0.01134	4002.1	2.31
C16(OH)	C6(C4:1-DC)	-0.601(-1.372,0.169)	0.01681	372.5	2.74
C3:1	C5	-0.014(-0.029,0.001)	0.00361	136.8	3.42
C14:2(OH)	C16:1(OH)	0.066(-0.01,0.143)	0.02177	105.8	-2.59
C18:1(OH)	C18:2	-0.097(-0.227,0.033)	0.03441	96.4	3.01
C5-DC(C6-OH)	Cit	-0.009(-0.014,-0.003)	0.00005	1452.6	10.54
C16:1(OH)	Carnosine	-0.161(-0.301,-0.02)	0.00396	628.4	3

C18:1(OH)	**Carnosine**	-0.429(-0.849,-0.008)	0.00824	219.8	3.24
C14:1(OH)	**Carnosine**	-0.095(-0.183,-0.008)	0.00863	204.7	2.64
C5:1-DC	**Cit**	-0.001(-0.002,0)	0.00005	198.6	16
C3-DC(C4-OH)	**Gly**	-0.001(-0.002,0)	0.04491	111	2.41
C16(OH)	**Carnosine**	-0.316(-0.643,0.011)	0.01473	101.7	2.2
C5	**Ile**	0.003(-0.001,0.007)	0.00634	83.2	-1.3
Thr	**Trp**	2.409(0.661,4.156)	0.0004	149.4	-2.54
PCaa40:5	**PCae34:2**	-0.081(-0.172,0.01)	0.01906	1430.4	1.74
PCaa34:1	**PCae34:1**	-1.831(-4.036,0.374)	0.02886	974.4	2.39
PCae36:3	**PCae36:5**	0.032(-0.001,0.064)	0.00813	570	-1.96
PCaa32:1	**PCae34:2**	-6.04(-13.609,1.528)	0.0399	565.3	1.86
PCaa40:5	**PCae36:2**	-0.246(-0.569,0.077)	0.05226	364.3	1.63
PCae36:3	**PCae38:6**	0.081(-0.013,0.174)	0.02002	178.5	-1.7
PCaa38:5	**PCae36:3**	-1.825(-3.992,0.343)	0.02138	163.4	2.41
PCae36:3	**PCae42:1**	0.697(-0.104,1.498)	0.02364	145	-2.1
PCaa32:3	**PCae36:3**	-0.779(-1.694,0.136)	0.03441	91	2.24
C3-DC(C4-OH)	**lysoPC20:4**	-0.122(-0.257,0.013)	0.00839	5222.8	2.94
C3-DC(C4-OH)	**lysoPC18:1**	-0.092(-0.191,0.007)	0.01047	4513.1	2.68
C5-DC(C6-OH)	**lysoPC18:1**	-0.002(-0.005,0.001)	0.05226	589.5	1.83
C5-DC(C6-OH)	**lysoPC16:1**	-0.012(-0.028,0.004)	0.04585	312.5	2.32
C3-DC(C4-OH)	**lysoPC18:2**	-0.065(-0.136,0.006)	0.00526	287.7	2.9
C5:1	**lysoPC14:0**	-0.004(-0.008,0.001)	0.02261	215	2.28
C5-DC(C6-OH)	**lysoPC18:2**	-0.002(-0.004,0)	0.00674	201.7	2.56
C14:1(OH)	**lysoPC18:2**	-0.002(-0.005,0)	0.00719	184.8	5.01
C18:1(OH)	**lysoPC18:2**	-0.009(-0.021,0.002)	0.00847	144.2	6.64
C16:1(OH)	**lysoPC18:2**	-0.004(-0.008,0.001)	0.00866	139.8	4.43
Ile	**lysoPC18:2**	-1.233(-2.532,0.065)	0.00373	176.4	3.34

Combination treatment cerebellum 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

Combination treatment plasma 4h

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Cit		40.56(10.598,70.522)	0.00001		-2.19
Sum of hexoses		13650(4364,22935)	0.00001		-2.4
C18:1(OH)		-0.013(-0.025,-0.001)	0.00012		6.18
C16(OH)		-0.012(-0.024,0.001)	0.00027		6.42
C3-DC(C4-OH)		-0.155(-0.351,0.04)	0.0009		5.5
C16:1(OH)		-0.007(-0.016,0.002)	0.00092		5.69
Thr		67.36(-6.182,140.902)	0.00124		-1.5
Pro		39.81(-7.795,87.415)	0.00144		-1.38
Ser		34.45(-2.212,71.112)	0.00202		-2.02

Met		13.64(-3.729,31.009)	0.00284		-1.39
C4:1		0.026(-0.006,0.058)	0.00334		-1.37
C7-DC		-0.006(-0.013,0.001)	0.00502		1.72
C16:1		-0.018(-0.041,0.005)	0.00524		1.99
C10		0.056(-0.025,0.136)	0.00558		-1.13
Spermidine		-3.46(-13.42,6.501)	0.00575		11.98
C14		-0.025(-0.058,0.007)	0.00585		2.31
Tyr		26.69(-3.025,56.405)	0.00671		-2.07
C16		-0.111(-0.262,0.04)	0.00684		3.2
C18:1		-0.087(-0.2,0.027)	0.00684		1.74
lysoPC14:0		0.895(-0.222,2.012)	0.00706		-1.84
Gly		36.24(-15.765,88.245)	0.00715		-1.35
C0		4.311(-1.421,10.043)	0.00762		-1.36
C16:2		-0.006(-0.015,0.003)	0.00883		3.18
C5-DC(C6-OH)		-0.006(-0.016,0.004)	0.01502		2.25
C12		-0.007(-0.018,0.004)	0.02001		1.28
C16:2(OH)		-0.003(-0.007,0.002)	0.0216		1.76
lysoPC18:2		63.2(-36.606,163.006)	0.02666		-1.15
lysoPC18:1		9.05(-5.744,23.844)	0.02688		-1.18
Phe		19.14(-16.227,54.507)	0.03111		-1.11
Lys		47.6(-41.545,136.745)	0.03177		-1.14
Asn		11.59(-9.183,32.363)	0.03555		-1.06
lysoPC17:0		0.346(-0.328,1.02)	0.03792		-0.93
C18:2		-0.036(-0.106,0.033)	0.0464		1.77
His		14.62(-14.593,43.833)	0.04921		-0.94
alpha.AAA		0.874(-0.865,2.613)	0.0403		-0.96
C3-DC(C4-OH)	**C5-OH(C3-DC-M)**	-2.342(-4.237,-0.447)	0.00005	1117711.3	7.69
C3-DC(C4-OH)	**C5**	-1.813(-3.436,-0.189)	0.00017	130513	6.99
C3-DC(C4-OH)	**C6(C4:1-DC)**	-2.6(-5.058,-0.141)	0.00023	128723.9	7.9
C10	**C18:1**	0.697(0.238,1.157)	0.00001	19011.1	-2.53
C3-DC(C4-OH)	**C8**	-1.516(-3.262,0.23)	0.0009	10860.9	4.54
C14:1	**C4:1**	-0.479(-0.761,-0.197)	0.00001	5714.9	4.28
C3-DC(C4-OH)	**C6:1**	-6.701(-15.13,1.729)	0.0016	4128.9	3.65
C10	**C14:1**	1.364(0.441,2.287)	0.00001	3769.1	-2.83
C3-DC(C4-OH)	**C5:1**	-4.126(-9.452,1.199)	0.00212	3566.4	3.75
C10	**C16:1**	2.255(0.513,3.997)	0.00005	844.8	-2.22
C3-DC(C4-OH)	**C4:1**	-3.023(-5.773,-0.273)	0.00005	734.7	6.2
C10	**C16**	0.617(0.2,1.035)	0.0001	607.7	-3.2
C10	**C14**	2.202(0.459,3.945)	0.0001	549.1	-2.23
C18:1	**C4:1**	-2.315(-3.801,-0.829)	0.00005	446.2	3.6
C14	**C4:1**	-0.676(-1.176,-0.175)	0.00005	386	3.61
C3-DC(C4-OH)	**C5-M-DC**	-5.767(-12.289,0.756)	0.00179	312.5	3.42
C5-DC(C6-OH)	**C5-OH(C3-DC-M)**	-0.101(-0.253,0.05)	0.0114	247.4	2.7
C16:1(OH)	**C4:1**	-0.161(-0.321,-0.001)	0.00001	234.4	11.29
C0	**C7-DC**	327.04(18.445,635.64)	0.00016	223.8	-1.76
C16	**C4:1**	-2.747(-4.999,-0.494)	0.0001	222.1	4.63
C10	**C18**	1.135(0.301,1.97)	0.00017	213.8	-3.03

C3-DC(C4-OH)	**C9**	-6.442(-17.444,4.56)	0.01304	190.1	2.32
C5-DC(C6-OH)	**C8**	-0.057(-0.153,0.038)	0.01388	171.9	1.92
C3-DC(C4-OH)	**C5:1-DC**	-13.645(-30.546,3.255)	0.00708	165.9	3.31
C16:1	**C4:1**	-0.538(-0.932,-0.145)	0.00012	148.7	3.31
C10	**C16:2**	9.794(2.111,17.477)	0.00027	109.7	-2.65
C3-DC(C4-OH)	**Kynurenine**	-0.156(-0.349,0.038)	0.0011	10226.6	4.29
C5-DC(C6-OH)	**Taurine**	0(0,0)	0.00048	4935.1	8.02
C3-DC(C4-OH)	**Trp**	-0.002(-0.005,0.001)	0.00059	4477	5.97
C3-DC(C4-OH)	**Orn**	-0.004(-0.009,0.001)	0.00057	4069.9	5.59
C3-DC(C4-OH)	**Gln**	0(-0.001,0)	0.00215	3688.3	4.01
C3-DC(C4-OH)	**Taurine**	-0.001(-0.001,0)	0.00068	2946.7	5.33
C3-DC(C4-OH)	**Val**	-0.001(-0.001,0)	0.00156	2575.8	3.71
C3-DC(C4-OH)	**His**	-0.003(-0.006,0.001)	0.00045	1114.4	5.03
C3-DC(C4-OH)	**Phe**	-0.002(-0.005,0.001)	0.00029	1114.1	6.58
C3-DC(C4-OH)	**Lys**	-0.001(-0.002,0)	0.00031	1070.3	5.7
C3-DC(C4-OH)	**Carnosine**	-0.633(-1.552,0.285)	0.00417	772.8	3.96
C3-DC(C4-OH)	**SDMA**	-0.311(-0.85,0.229)	0.00454	678.9	6.16
C3-DC(C4-OH)	**Creatinine**	-0.017(-0.042,0.008)	0.00418	629.5	4.71
C3-DC(C4-OH)	**DOPA**	-0.431(-1.362,0.499)	0.00717	574.8	5.66
C3-DC(C4-OH)	**Ile**	-0.001(-0.002,0)	0.0046	546	3.27
C5-DC(C6-OH)	**SDMA**	-0.015(-0.036,0.006)	0.00603	440.4	4.12
C3-DC(C4-OH)	**Gly**	-0.002(-0.004,0)	0.00016	400.4	6.9
C5-DC(C6-OH)	**Kynurenine**	-0.006(-0.014,0.002)	0.01133	295.5	2.07
C3-DC(C4-OH)	**PEA**	-1.71(-5.347,1.926)	0.00992	287	4.6
C3-DC(C4-OH)	**Leu**	0(-0.001,0)	0.01193	248.2	4.04
C5-DC(C6-OH)	**Orn**	0(0,0)	0.00388	207.8	3.93
C3-DC(C4-OH)	**MetSO**	-0.233(-0.547,0.081)	0.00169	206.1	5.68
C16	**Tyr**	-0.004(-0.009,0)	0.00023	186.1	6.47
C16:2	**Tyr**	0(-0.001,0)	0.00023	173.1	5.91
C5-DC(C6-OH)	**DOPA**	-0.02(-0.062,0.022)	0.02329	104.2	2.91
C5-DC(C6-OH)	**Lys**	0(0,0)	0.00145	100.8	4.43
Glu	**Orn**	-0.762(-1.718,0.193)	0.00114	1241.3	4.48
Orn	**Spermidine**	35.353(13.312,57.395)	0.00012	434	-3.14
Asp	**Spermidine**	4.931(0.91,8.952)	0.00012	345.6	-2.17
His	**Putrescine**	39.844(-5.689,85.377)	0.00214	105.3	-1.72
Asp	**Orn**	-0.099(-0.245,0.048)	0.00607	104	2.89
PCaa36:5	**PCaa38:5**	0.017(0.007,0.026)	0.00001	5123514.3	-3.57
PCaa32:0	**PCae36:2**	-0.187(-0.294,-0.079)	0.00001	1403510.9	3.39
PCaa36:4	**PCaa40:5**	4.544(1.914,7.173)	0.00001	505676.8	-4.74
PCae38:4	**PCae40:6**	0.266(0.054,0.477)	0.00023	45679.4	-3.43
PCae40:1	**PCae40:5**	0.536(0.057,1.015)	0.00044	11873.4	-2
PCaa36:5	**PCaa40:5**	0.143(0.026,0.261)	0.00023	8103.4	-2.47
PCae40:1	**PCae40:6**	0.295(0.017,0.573)	0.00086	5903.4	-2.69
PCaa32:1	**PCaa36:5**	-0.096(-0.186,-0.006)	0.00069	4579.2	2.59
PCae42:1	**PCae42:3**	-0.188(-0.364,-0.011)	0.00069	4455.6	2.58
PCaa40:6	**PCae40:1**	-3.246(-6.255,-0.236)	0.00088	3588.2	2.6
PCaa36:4	**PCaa40:6**	0.666(0.011,1.321)	0.0009	3399.1	-2.08

PCaa38:4	**PCaa40:6**	0.673(0.015,1.331)	0.00107	2652.2	-3.07
PCaa42:5	**PCae42:3**	-0.079(-0.158,-0.001)	0.00115	2119.1	2.01
PCae38:4	**PCae40:5**	0.49(-0.002,0.983)	0.00148	1842	-3.19
PCaa38:4	**PCaa40:5**	4.761(0.384,9.138)	0.00069	1568.1	-2.47
PCaa40:5	**PCae36:2**	-0.339(-0.653,-0.024)	0.00069	1531.3	2.7
PCaa40:6	**PCae38:4**	-1.399(-2.862,0.064)	0.00152	1522.8	2.13
PCaa36:5	**PCaa40:6**	0.022(0,0.044)	0.00147	1428.5	-2.07
PCaa42:1	**PCae32:2**	-0.187(-0.376,0.001)	0.00072	1158.9	4.17
PCaa36:5	**PCae40:6**	0.55(0.004,1.096)	0.00178	1059.4	-2.64
PCaa40:6	**PCae42:3**	-15.532(-32.041,0.976)	0.00208	952.3	1.99
PCae40:5	**PCae42:3**	-0.312(-0.655,0.032)	0.00239	882.6	1.66
PCaa36:5	**PCae40:5**	0.954(0.028,1.879)	0.00209	827.1	-4.1
PCaa42:4	**PCae42:3**	-0.078(-0.167,0.01)	0.00237	725.6	2.4
PCaa36:5	**PCae38:5**	0.318(-0.022,0.658)	0.00229	710.1	-2.54
PCaa34:4	**PCaa40:5**	0.023(0.002,0.043)	0.00142	494.1	-2.78
PCaa38:0	**PCae42:3**	-0.575(-1.208,0.057)	0.00208	477.9	2.15
PCaa36:5	**PCaa38:6**	0.008(-0.001,0.018)	0.00301	475.8	-2.14
PCaa36:6	**PCae40:5**	0.07(-0.012,0.151)	0.00388	417.5	-2.18
PCaa38:6	**PCae40:1**	-5.834(-13.43,1.763)	0.00546	413.5	4.55
PCaa36:5	**PCae36:4**	0.273(-0.026,0.573)	0.00334	408	-2.87
PCaa34:4	**PCaa38:0**	0.15(-0.027,0.327)	0.00243	377.8	-1.58
PCaa32:2	**PCaa40:5**	0.016(-0.001,0.034)	0.0017	372.9	-1.7
PCaa42:1	**PCae42:3**	-0.054(-0.11,0.002)	0.0015	365.4	2.73
PCaa36:6	**PCae34:1**	0.043(-0.006,0.093)	0.00302	363	-1.93
PCaa34:4	**PCae34:1**	0.098(-0.018,0.215)	0.00306	357.1	-1.74
PCae40:6	**PCae42:3**	-0.493(-1.124,0.137)	0.00536	349.6	1.76
PCaa36:6	**PCaa40:5**	0.01(-0.001,0.022)	0.00182	337.5	-1.88
PCaa32:0	**PCaa34:4**	-2.136(-4.691,0.419)	0.00338	334.1	1.8
PCaa34:4	**PCae44:5**	1.897(-0.373,4.167)	0.00341	329.9	-1.8
PCaa32:3	**PCaa34:4**	-0.043(-0.095,0.008)	0.00347	322.2	3.16
PCaa34:4	**PCaa42:5**	0.832(-0.247,1.911)	0.00354	313.6	-1.32
PCae32:2	**PCae42:1**	0.061(-0.011,0.133)	0.00399	293	-1.94
PCaa34:4	**PCaa42:4**	0.883(-0.226,1.992)	0.00396	257.2	-1.49
PCaa36:6	**PCaa40:6**	0.002(0,0.004)	0.00502	245.5	-1.59
PCaa36:5	**PCae34:1**	0.585(-0.07,1.241)	0.00395	238.8	-2.6
PCae34:1	**PCae40:1**	-0.114(-0.25,0.023)	0.00399	234.3	1.67
PCaa36:4	**PCae40:6**	15.958(-5.018,36.934)	0.00719	222.2	-1.83
PCaa42:5	**PCae40:1**	-0.017(-0.037,0.004)	0.00502	221.3	1.7
PCae34:2	**PCae34:3**	-0.419(-0.937,0.099)	0.00537	195.9	1.62
PCaa34:3	**PCaa40:5**	0.241(-0.045,0.526)	0.00267	188.7	-1.48
PCaa34:4	**PCae40:5**	0.16(-0.043,0.362)	0.00523	169.4	-1.72
PCaa34:3	**PCaa40:6**	0.034(-0.011,0.078)	0.00648	164.8	-1.38
PCae34:3	**PCae38:6**	0.075(-0.024,0.174)	0.00606	162.8	-1.56
PCaa38:0	**PCae40:1**	-0.122(-0.27,0.026)	0.00421	159.5	2.18
PCaa36:6	**PCaa42:5**	0.361(-0.119,0.841)	0.00665	142.9	-1.48
PCaa36:4	**PCae40:5**	28.23(-8.336,64.796)	0.00808	136.4	-2.8
PCaa38:4	**PCae40:5**	28.71(-9.559,66.979)	0.0083	131.3	-3.18

PCaa38:0	**PCae42:4**	-1.283(-2.864,0.299)	0.00497	126.5	1.65
PCaa42:4	**PCae40:1**	-0.016(-0.038,0.006)	0.00783	114.8	2.19
PCaa38:1	**PCae38:1**	-0.295(-0.674,0.083)	0.00592	111.3	1.45
PCaa36:6	**PCae40:6**	0.039(-0.015,0.093)	0.01055	107.4	-1.87
PCae42:3	**PCae44:3**	2.145(-0.736,5.025)	0.00563	105.2	-1.75
PCae40:4	**PCae40:5**	0.19(-0.081,0.461)	0.00981	103.2	-1.6
PCaa32:0	**PCaa34:2**	-0.002(-0.005,0.001)	0.00808	99.9	2.5
PCaa38:4	**PCae40:6**	15.724(-7.22,38.668)	0.01279	96.8	-2.59
PCaa34:4	**SM24:0**	0.014(0.001,0.027)	0.00059	4902.7	-3.39
PCae34:3	**SM24:0**	0.013(-0.001,0.027)	0.00121	1808.9	-1.71
PCaa34:4	**SM16:1**	0.031(0,0.062)	0.00148	748.7	-3.64
PCae34:3	**SM(OH)22:1**	0.045(-0.007,0.097)	0.00208	646.1	-2.85
PCae34:3	**SM16:1**	0.029(-0.003,0.062)	0.00198	482.9	-1.88
PCaa34:4	**SM(OH)22:1**	0.049(-0.005,0.103)	0.00388	248.9	-3.58
PCaa36:6	**SM24:0**	0.006(-0.002,0.014)	0.00574	165.2	-2.05
PCaa34:4	**SM16:0**	0.004(-0.001,0.01)	0.00643	122.4	-3.25
PCae36:2	**SM24:0**	0.091(-0.03,0.211)	0.00748	110.5	-1.5
PCae34:3	**SM16:0**	0.004(-0.002,0.01)	0.00846	98.9	-2.09
PCaa36:6	**SM(OH)22:1**	0.022(-0.006,0.051)	0.00713	97.7	-2.38
PCaa36:5	**SM(OH)22:1**	0.31(-0.072,0.692)	0.00715	97.2	-2.11
lysoPC17:0	**PCae34:1**	0.224(0.024,0.424)	0.00058	544.5	-2.17
lysoPC17:0	**PCae38:6**	0.188(0.022,0.354)	0.00076	346.4	-3.68
lysoPC17:0	**PCae34:2**	0.132(0.005,0.259)	0.00111	196.7	-2.68
lysoPC17:0	**PCaa40:5**	0.049(0.003,0.095)	0.00115	186.2	-3.5
lysoPC18:0	**PCae34:1**	19.421(-0.385,39.227)	0.0018	151.3	-2.14
lysoPC28:1	**PCaa42:1**	1.451(-0.301,3.203)	0.003	128.9	-1.5
lysoPC17:0	**PCae44:5**	4.305(0.06,8.55)	0.00158	113.2	-2.2
lysoPC16:1	**PCae34:1**	0.247(-0.071,0.565)	0.00661	108.3	-2.62
lysoPC18:0	**SM24:0**	2.868(0.919,4.817)	0.00005	33326.8	-3.04
lysoPC17:0	**SM24:0**	0.033(0.009,0.057)	0.0001	7449.3	-2.82
lysoPC17:0	**SM16:1**	0.072(0.016,0.127)	0.00017	3477.3	-2.88
lysoPC18:0	**SM16:1**	6.247(0.958,11.536)	0.00038	1685.8	-3.03
lysoPC16:0	**SM16:1**	7.899(-0.41,16.207)	0.00189	491.9	-2.12
lysoPC16:0	**SM24:0**	3.469(-0.416,7.355)	0.00288	258.8	-2.02
lysoPC17:0	**SM(OH)22:1**	0.114(-0.006,0.235)	0.00099	234.6	-1.93
lysoPC17:0	**SM(OH)22:2**	0.257(-0.009,0.523)	0.00101	228	-1.65
lysoPC18:0	**SM24:1**	3.661(-0.118,7.441)	0.00148	204.4	-2.04
C3-DC(C4-OH)	**PCae36:3**	-0.105(-0.218,0.008)	0.00038	54037.1	4.07
C10	**PCae34:1**	0.033(0.014,0.052)	0.00001	32387.2	-3.05
C3-DC(C4-OH)	**PCae36:0**	-0.524(-1.113,0.065)	0.00025	31285.5	4.91
C3-DC(C4-OH)	**PCaa36:2**	0(-0.001,0)	0.00044	27999.2	4.83
C3-DC(C4-OH)	**PCaa34:2**	0(-0.001,0)	0.0005	27155.8	4.89
C3-DC(C4-OH)	**PCae36:2**	-0.022(-0.046,0.003)	0.00031	20612.1	4.88
C3-DC(C4-OH)	**PCaa30:0**	-0.185(-0.397,0.027)	0.00072	20491	3.64
C3-DC(C4-OH)	**PCaa34:4**	-0.187(-0.379,0.005)	0.00027	16358.6	3.98
C3-DC(C4-OH)	**PCaa36:3**	-0.001(-0.003,0)	0.00058	14965.8	4.44
C3-DC(C4-OH)	**PCae38:3**	-0.094(-0.198,0.01)	0.00093	13527.1	3.26

C3-DC(C4-OH)	**PCaa28:1**	-0.283(-0.586,0.02)	0.00069	12092.7	2.81
C3-DC(C4-OH)	**PCae34:3**	-0.198(-0.418,0.023)	0.00038	11672.9	3.89
C3-DC(C4-OH)	**PCae38:2**	-0.037(-0.078,0.003)	0.00075	10034.6	3.78
C3-DC(C4-OH)	**PCaa36:1**	-0.003(-0.006,0)	0.00053	9448	5.32
C3-DC(C4-OH)	**PCae34:0**	-0.712(-1.555,0.13)	0.00121	8941.9	3.44
C3-DC(C4-OH)	**PCae42:4**	-0.543(-1.127,0.041)	0.00115	8901.4	2.91
C3-DC(C4-OH)	**PCae44:6**	-0.847(-1.815,0.121)	0.00103	8290	3.18
C3-DC(C4-OH)	**PCaa32:3**	-0.669(-1.422,0.085)	0.00094	8130.1	2.82
C3-DC(C4-OH)	**PCae44:4**	-1.201(-2.646,0.245)	0.0014	7475.4	3.23
C3-DC(C4-OH)	**PCae32:2**	-0.715(-1.484,0.053)	0.00127	7179.1	2.82
C3-DC(C4-OH)	**PCaa36:6**	-0.358(-0.772,0.057)	0.00069	7061.2	3.5
C3-DC(C4-OH)	**PCae38:4**	-0.026(-0.057,0.006)	0.00138	6877.4	3.01
C3-DC(C4-OH)	**PCaa38:4**	-0.001(-0.001,0)	0.00115	6867.2	3.52
C3-DC(C4-OH)	**PCae36:1**	-0.058(-0.126,0.009)	0.0014	6814.8	3.03
C3-DC(C4-OH)	**PCaa36:4**	-0.001(-0.001,0)	0.00103	6423.7	3.83
C3-DC(C4-OH)	**PCae38:1**	-0.144(-0.306,0.018)	0.00108	6362.6	4.17
C3-DC(C4-OH)	**PCaa34:1**	-0.001(-0.003,0)	0.00133	6190.9	3.95
C3-DC(C4-OH)	**PCaa42:2**	-0.657(-1.428,0.115)	0.00155	5195.2	3.27
C3-DC(C4-OH)	**PCae36:5**	-0.073(-0.169,0.023)	0.0014	5011.6	3.58
C3-DC(C4-OH)	**PCaa32:2**	-0.172(-0.398,0.054)	0.00138	4811.7	3.1
C3-DC(C4-OH)	**PCae40:1**	-0.041(-0.089,0.008)	0.00116	4412.7	2.98
C3-DC(C4-OH)	**PCae40:4**	-0.069(-0.155,0.018)	0.00179	4398.5	2.86
C3-DC(C4-OH)	**PCaa34:3**	-0.01(-0.024,0.004)	0.0018	4048.2	3.09
C3-DC(C4-OH)	**PCaa32:0**	-0.016(-0.035,0.004)	0.00088	3021.9	4.07
C3-DC(C4-OH)	**PCae38:0**	-0.055(-0.13,0.019)	0.00242	2869.4	2.62
C3-DC(C4-OH)	**PCae42:3**	-0.185(-0.403,0.034)	0.00144	2673.8	2.79
C3-DC(C4-OH)	**PCae38:6**	-0.049(-0.107,0.009)	0.00125	2624.2	3
C3-DC(C4-OH)	**PCae42:5**	-0.213(-0.48,0.053)	0.00141	2401.9	3.54
C3-DC(C4-OH)	**PCaa26:0**	-0.154(-0.327,0.02)	0.00211	2325.4	3.01
C3-DC(C4-OH)	**PCaa36:5**	-0.029(-0.068,0.011)	0.00108	2287	3.83
C3-DC(C4-OH)	**PCae30:0**	-0.731(-1.557,0.095)	0.00201	2066.6	2.59
C3-DC(C4-OH)	**PCae40:2**	-0.209(-0.469,0.05)	0.00266	2050.9	2.5
C3-DC(C4-OH)	**PCae32:1**	-0.276(-0.622,0.069)	0.00286	1973.3	2.33
C3-DC(C4-OH)	**PCaa40:2**	-0.21(-0.459,0.04)	0.00254	1849	2.66
C3-DC(C4-OH)	**PCaa38:5**	-0.003(-0.007,0.002)	0.00324	1845.1	2.9
C3-DC(C4-OH)	**PCaa38:3**	-0.002(-0.004,0.001)	0.00318	1712.9	2.88
C3-DC(C4-OH)	**PCae36:4**	-0.029(-0.066,0.008)	0.00219	1316.7	2.83
C3-DC(C4-OH)	**PCaa40:1**	-0.344(-0.76,0.071)	0.00201	1108.7	2.92
C3-DC(C4-OH)	**PCae40:5**	-0.091(-0.204,0.023)	0.00207	1094.7	2.51
C3-DC(C4-OH)	**PCaa38:6**	-0.001(-0.002,0)	0.00356	965.2	2.52
C3-DC(C4-OH)	**PCae40:6**	-0.057(-0.133,0.019)	0.00292	894.5	2.52
C3-DC(C4-OH)	**PCae44:5**	-1.04(-2.334,0.254)	0.00183	878.1	2.6
C3-DC(C4-OH)	**PCae34:1**	-0.055(-0.123,0.013)	0.00173	852.3	2.96
C3-DC(C4-OH)	**PCae30:2**	-1.099(-2.623,0.425)	0.00485	767.3	2.45
C3-DC(C4-OH)	**PCaa42:6**	-0.189(-0.448,0.069)	0.0034	709.8	3.01
C3-DC(C4-OH)	**PCae40:3**	-0.139(-0.32,0.042)	0.00403	693.9	2.18
C3-DC(C4-OH)	**PCaa40:4**	-0.032(-0.076,0.013)	0.00397	597.2	2.8

C3-DC(C4-OH)	**PCae42:2**	-0.222(-0.522,0.078)	0.00444	594.3	2.18
C3-DC(C4-OH)	**PCae42:0**	-0.2(-0.469,0.069)	0.00318	543.3	2.92
C3-DC(C4-OH)	**PCae38:5**	-0.027(-0.064,0.009)	0.00285	506.1	2.8
C10	**PCae42:5**	0.1(0.022,0.178)	0.0001	487.3	-2.13
C3-DC(C4-OH)	**PCaa40:6**	-0.002(-0.005,0.001)	0.00369	398.1	2.58
C3-DC(C4-OH)	**PCaa38:0**	-0.06(-0.134,0.014)	0.00264	336.6	2.45
C10	**PCaa32:0**	0.008(0.002,0.014)	0.00012	317.2	-2.86
C3-DC(C4-OH)	**PCaa42:5**	-0.394(-0.933,0.144)	0.00396	316.8	2.29
C3-DC(C4-OH)	**PCaa42:4**	-0.415(-0.978,0.147)	0.00405	313	2.26
C3-DC(C4-OH)	**PCaa42:0**	-0.637(-1.527,0.253)	0.00531	308	2.21
C10	**PCae36:3**	0.037(0.008,0.065)	0.00016	252.7	-2.75
C10	**PCaa40:5**	0.007(0.002,0.013)	0.00016	241.5	-2.57
C10	**PCae38:6**	0.028(0.006,0.049)	0.00017	206	-3.17
C3-DC(C4-OH)	**PCaa40:3**	-0.149(-0.368,0.07)	0.00963	184.6	2.2
C4:1	**PCae34:1**	0.015(0.002,0.028)	0.00012	179.5	-1.85
C10	**PCae38:5**	0.017(0.004,0.03)	0.00021	152.6	-2.98
C5-DC(C6-OH)	**PCaa36:5**	-0.001(-0.003,0)	0.0064	147.6	2.67
C5-DC(C6-OH)	**PCae36:0**	-0.023(-0.055,0.009)	0.00843	140.9	2.14
C5-DC(C6-OH)	**PCae42:3**	-0.009(-0.021,0.003)	0.01011	137.9	2.14
C5-DC(C6-OH)	**PCae32:2**	-0.033(-0.084,0.018)	0.01763	136.5	2.43
C5-DC(C6-OH)	**PCaa36:6**	-0.017(-0.038,0.005)	0.00896	136	2.42
C5-DC(C6-OH)	**PCae40:1**	-0.002(-0.004,0.001)	0.01165	135.6	1.96
C5-DC(C6-OH)	**PCae38:0**	-0.002(-0.006,0.001)	0.02017	119.6	1.68
C5-DC(C6-OH)	**PCae38:4**	-0.001(-0.003,0.001)	0.02041	118.1	1.78
C5-DC(C6-OH)	**PCae40:4**	-0.003(-0.007,0.002)	0.02089	110.6	1.63
C5-DC(C6-OH)	**PCae36:3**	-0.004(-0.012,0.003)	0.02307	109.5	1.68
C5-DC(C6-OH)	**PCaa38:4**	0(0,0)	0.01822	106.7	2.06
C5-DC(C6-OH)	**PCaa34:3**	0(-0.001,0)	0.02115	101.1	2.2
C10	**PCae34:2**	0.021(0.003,0.039)	0.00027	100.2	-2.14
C3-DC(C4-OH)	**SM16:0**	-0.003(-0.007,0.001)	0.00093	7740	2.73
C3-DC(C4-OH)	**SM(OH)14:1**	-0.046(-0.101,0.009)	0.00132	5101.1	2.35
C3-DC(C4-OH)	**SM18:1**	-0.092(-0.207,0.022)	0.00186	3052.6	3.12
C3-DC(C4-OH)	**SM(OH)24:1**	-0.518(-1.217,0.181)	0.0027	2024.6	2.66
C3-DC(C4-OH)	**SM(OH)16:1**	-0.301(-0.691,0.09)	0.00268	1272.8	2.29
C10	**SM24:0**	0.005(0.001,0.008)	0.00005	1178.4	-2.51
C3-DC(C4-OH)	**SM24:0**	-0.008(-0.019,0.002)	0.00194	877.3	2.92
C3-DC(C4-OH)	**SM18:0**	-0.036(-0.087,0.014)	0.00382	834.8	3.02
C3-DC(C4-OH)	**SM24:1**	-0.01(-0.022,0.002)	0.00212	782.4	3.19
C3-DC(C4-OH)	**SM(OH)22:2**	-0.066(-0.156,0.024)	0.00328	663.2	2.39
C3-DC(C4-OH)	**SM16:1**	-0.015(-0.034,0.004)	0.00189	520.5	2.54
C16(OH)	**SM18:1**	-0.007(-0.011,-0.003)	0.00001	343.9	6.27
C3-DC(C4-OH)	**SM(OH)22:1**	-0.022(-0.054,0.01)	0.00428	210.1	1.97
C10	**SM24:1**	0.006(0.001,0.011)	0.00023	139.2	-2.91
C10	**SM16:1**	0.01(0.002,0.019)	0.00023	131.3	-2.37
C3-DC(C4-OH)	**lysoPC26:1**	-0.083(-0.19,0.024)	0.0014	5419.1	4.43
C3-DC(C4-OH)	**lysoPC16:0**	-0.001(-0.002,0)	0.00041	5191.4	5.88
C3-DC(C4-OH)	**lysoPC18:2**	-0.001(-0.003,0)	0.0001	4544.3	9.18

C3-DC(C4-OH)	**lysoPC20:4**	-0.004(-0.009,0)	0.00059	3346.5	6.66
C3-DC(C4-OH)	**lysoPC18:0**	-0.001(-0.003,0)	0.00025	3076.6	5.76
C3-DC(C4-OH)	**lysoPC16:1**	-0.056(-0.128,0.016)	0.00138	2172.8	4.01
C3-DC(C4-OH)	**lysoPC17:0**	-0.112(-0.241,0.016)	0.00025	1908.2	5.35
C3-DC(C4-OH)	**lysoPC24:0**	-0.187(-0.427,0.053)	0.00265	1794.1	2.67
C3-DC(C4-OH)	**lysoPC20:3**	-0.02(-0.041,0)	0.00117	1768	6.72
C3-DC(C4-OH)	**lysoPC18:1**	-0.006(-0.012,0)	0.00021	1598	7.91
C3-DC(C4-OH)	**lysoPC28:1**	-0.321(-0.683,0.04)	0.00254	1244.5	2.58
C3-DC(C4-OH)	**lysoPC28:0**	-0.192(-0.425,0.042)	0.00471	1064.8	2.27
C16	**lysoPC16:0**	-0.001(-0.001,0)	0.00016	396.5	4.78
C18:1	**lysoPC18:0**	-0.001(-0.001,0)	0.00016	363.6	3.21
C16	**lysoPC17:0**	-0.091(-0.164,-0.018)	0.00017	271.9	4.2
C18:1	**lysoPC18:1**	-0.004(-0.008,-0.001)	0.0002	239.1	4.24
C18:1	**lysoPC17:0**	-0.077(-0.136,-0.018)	0.00021	209	3.02
C16:2	**lysoPC16:0**	0(0,0)	0.00027	199.9	3.89
C3-DC(C4-OH)	**lysoPC26:0**	-0.198(-0.46,0.064)	0.01127	197.2	1.69
C14:1	**lysoPC17:0**	-0.014(-0.028,-0.001)	0.00086	173.6	2.75
C18	**lysoPC18:0**	0(0,0)	0.00194	135.3	2.57
C5-DC(C6-OH)	**lysoPC26:1**	-0.003(-0.009,0.003)	0.01625	134.7	2.32
C18:1	**lysoPC18:2**	-0.001(-0.002,0)	0.00029	125.4	3.9
C16:2	**lysoPC17:0**	-0.005(-0.01,0)	0.00041	113	3.78
C5-DC(C6-OH)	**lysoPC16:1**	-0.002(-0.007,0.002)	0.00967	110.6	2.35
C18:1	**lysoPC16:0**	0(-0.001,0)	0.00031	110.4	3.2

6.1.2. Sub-chronic treatment

Significant changes in single metabolite concentration and in metabolite ratios during

Metformin

Metformin Testis 14 d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Lys		-22.23 (-42.97,-1.49)	0.024		6.34
Pro		-31.7 (-62.53,-0.87)	0.041		3.51
C18:2	**Sum of hexoses**	-0.0003 (-0.0006,0.0)	0.041	136.9	4.39

Metformin adrenal gland 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
C4	**MetSO**	0.678 (0.106,1.249)	0.012	2027.8	-2.94
C2	**MetSO**	15.003 (2.063,27.943)	0.054	200.4	-3.13
C5-DC(C6-OH)	**MetSO**	0.087 (-0.009,0.182)	0.054	148.7	-2.23
C5.OH(C3.DC.M)	**MetSO**	0.122 (-0.018,0.262)	0.173	28.1	-2.30

Metformin lung 14 d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
C16(OH)		-0.172(-0.317,-0.027)	0.00322		4.05
C14:1(OH)		-0.033(-0.064,-0.002)	0.00376		3.51
C16:1(OH)		-0.067(-0.129,-0.004)	0.00376		4.41
C7(DC)		-0.018(-0.035,-0.001)	0.00383		3.32
C18:1(OH)		-0.182(-0.361,-0.004)	0.00598		3.11
C14:1		-0.04(-0.08,0.001)	0.00681		2.61
C14:2		-0.017(-0.037,0.003)	0.01124		3.14
C16:1		-0.112(-0.248,0.024)	0.01149		2.92
C14:2(OH)		-0.013(-0.028,0.001)	0.01454		2.79
C14		-0.107(-0.25,0.036)	0.02144		1.71
C5-DC(C6-OH)		-0.02(-0.047,0.007)	0.02221		2.52
C16:2(OH)		-0.017(-0.038,0.005)	0.02611		2.8
C16:2		-0.026(-0.067,0.014)	0.03493		1.97
C16		-0.435(-1.084,0.214)	0.03529		2.1
C12:1		-0.016(-0.041,0.01)	0.03672		1.1
C2		-14.942(-39.461,9.576)	0.04651		2.11
C5-DC(C6-OH)	**C5.OH(C3.DC.M)**	-0.136(-0.235,-0.037)	3.00E-04	128922	9.27
C2	**C5.OH(C3.DC.M)**	-95.283(-175.141,-15.425)	0.00215	3603.4	6.16
C5.OH(C3.DC.M)	**C6(C4:1(DC))**	0.647(0.107,1.188)	0.00322	1396.9	-2.2
C14:2(OH)	**C5.OH(C3.DC.M)**	-0.084(-0.143,-0.025)	0.00145	490.8	4.8
C18:1(OH)	**C5.OH(C3.DC.M)**	-1.085(-1.792,-0.379)	3.00E-04	305	4.6
C5.OH(C3.DC.M)	**C7(DC)**	2.995(0.936,5.054)	3.00E-04	292.7	-2.54
C16(OH)	**C5.OH(C3.DC.M)**	-0.978(-1.522,-0.434)	3.00E-04	244	5
C5-DC(C6-OH)	**C5.M.DC**	-1.93(-4.394,0.535)	0.02206	153.7	3.59
C14:1(OH)	**C5.OH(C3.DC.M)**	-0.196(-0.314,-0.077)	3.00E-04	147.1	4.64
C5-DC(C6-OH)	**C8**	-0.146(-0.345,0.053)	0.02525	146.6	2.23
C16:1(OH)	**C5.OH(C3.DC.M)**	-0.387(-0.639,-0.135)	3.00E-04	145.9	5.8
C16:2(OH)	**C5.OH(C3.DC.M)**	-0.103(-0.19,-0.015)	0.00374	117.5	3.37
C18:2	**C5.OH(C3.DC.M)**	-1.963(-4.04,0.114)	0.00723	103.4	3.25
C5.OH(C3.DC.M)	**C8**	0.421(-0.202,1.043)	0.02953	99.5	-1.26
C14:2(OH)	**C4:1**	-1.033(-1.943,-0.123)	0.00327	49.1	3.68
C5-DC(C6-OH)	**Gln**	0(0,0)	0.01459	796.6	3.34
C5.OH(C3.DC.M)	**Trp**	0.008(0.001,0.015)	0.00545	781.9	-2.53
C5.OH(C3.DC.M)	**Val**	0.001(0,0.003)	0.0082	672.2	-2.22
C5-DC(C6-OH)	**Gly**	0(0,0)	0.02884	374.1	2.63
C5-DC(C6-OH)	**Sarcosine**	-0.006(-0.014,0.002)	0.02195	334.2	2.22
C5.OH(C3.DC.M)	**Ile**	0.002(-0.001,0.005)	0.01873	268.8	-1.41
C5.OH(C3.DC.M)	**Phe**	0.003(0,0.007)	0.00888	239.7	-1.76
C5.OH(C3.DC.M)	**Leu**	0.001(0,0.002)	0.02648	237.4	-1.71
C6(C4:1(DC))	**Gln**	0(0,0)	0.03402	124.6	1.91
C5-DC(C6-OH)	**Leu**	0(-0.001,0)	0.04247	86.6	2.73
C5-DC(C6-OH)	**Orn**	-0.003(-0.008,0.002)	0.03963	85	1.44

PCaa32:2	**PCae38:0**	1.889 (0.144,3.634)	0.0050	975.985558	-2.48435476
PCaa32:2	**PCaa32:3**	0.814 (0.074,1.554)	0.0055	777.201363	-3.13499242
PCaa32:3	**PCaa34:3**	-0.062 (-0.127,0.003)	0.0064	1174.11001	3.83081603
PCaa34:3	**PCae38:0**	1.06 (-0.183,2.302)	0.0143	238.076641	-1.76903263
PCaa34:2	**PCaa36:4**	0.204 (-0.028,0.436)	0.0124	162.014316	-1.80497966
PCaa38:3	**PCaa40:4**	0.302 (-0.108,0.711)	0.0251	153.390233	-1.42725896
PCaa34:2	**PCae36:5**	2.193 (-0.4,4.785)	0.0127	151.516728	-1.52791975
PCae36:3	**PCae38:5**	0.059 (-0.011,0.129)	0.0127	112.482391	-1.55204921
PCae36:3	**PCae40:6**	0.468 (-0.113,1.049)	0.0147	84.8141594	-1.63000698
PCaa38:3	**PCae38:4**	0.427 (-0.159,1.014)	0.0331	80.9408621	-1.85121249
C3-DC (C4-OH	**lysoPC18:2**	0.006 (0.001,0.01)	0.0036	881.772794	-3.17231691
C5.OH(C3.DC.M)	**lysoPC18:1**	0.013 (0.002,0.025)	0.0048	440.499155	-3.00944897
C5.OH(C3.DC.M)	**lysoPC20:4**	0.018 (0.001,0.036)	0.0070	222.393152	-2.49251001

Metformin Cerebellum 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
PCaa34:3	**PCaa36:5**	0.149(0.004,0.294)	0.12888	3259.8	-2.18
PCae34:2	**PCae38:2**	0.26(0.008,0.512)	0.12888	1420.5	-2.26
PCaa34:3	**PCaa38:5**	0.008(-0.001,0.016)	0.14961	579.6	-1.94
PCae42:1	**PCae44:5**	0.088(-0.001,0.178)	0.12888	543.3	-2.08
MetSO	**PCaa38:3**	-0.007(-0.012,-0.003)	0.05566	171.7	8.55
MetSO	**PCae42:1**	-0.017(-0.034,-0.001)	0.05566	74.9	10.04
MetSO	**PCaa34:2**	-0.001(-0.001,0)	0.05566	49.9	6.43
C5:1	**MetSO**	0.133(0.032,0.234)	0.05566	44.4	-5.69
MetSO	**PCaa34:3**	-0.022(-0.042,-0.003)	0.12888	6.5	4.86
MetSO	**PCae40:6**	-0.025(-0.045,-0.005)	0.12888	6.1	4.55
MetSO	**PCae38:4**	-0.029(-0.056,-0.002)	0.13049	2.7	3.91

Metformin plasma 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
PCae42:4		-0.108(-0.259,0.042)	0.02438		3.08
PCaa32:1		-0.798(-1.951,0.355)	0.03332		1.66
PCae44:4		-0.046(-0.115,0.023)	0.03784		2.61
C16(OH)		-0.003(-0.008,0.001)	0.041		1.56
SM(OH)22:1		1.394(-0.738,3.526)	0.041		-1.43
Gln	**His**	1.295(-0.309,2.9)	0.02364	162.2	-1.95
Lys	**Ser**	-0.287(-0.625,0.051)	0.01212	78.4	2.82
C18:2	**C4**	0.066(0.002,0.13)	0.00596	2174.6	-2.14
C18:1	**C4**	0.109(0,0.218)	0.00723	1967.3	-2.1
C16	**C4**	0.112(-0.023,0.247)	0.01212	642.8	-1.6
C5	**C5.OH(C3.DC.M)**	-0.7(-1.414,0.014)	0.00507	554.5	3.03
C18:1	**C18:1(OH)**	4.131(-0.709,8.97)	0.0163	413.8	-1.7
C2	**C4**	3.134(-0.825,7.092)	0.02027	246.5	-2.15

C10	**C16(OH)**	9.313(1.027,17.6)	0.00352	239.4	-2.04
C16	**C18:1(OH)**	4.143(-1.205,9.492)	0.02387	222	-1.47
C14:2	**C4**	0.006(-0.002,0.013)	0.02603	156.1	-1.45
C18:1	**C6(C4:1(DC))**	0.974(-0.27,2.218)	0.02919	137.9	-1.81
C3-DC (C4-OH)	**C4**	0.1(-0.039,0.239)	0.02886	123.4	-1.34
C18:1(OH)	**C18:2**	-0.068(-0.162,0.026)	0.02616	111.5	2.72
C16:1	**C4**	0.023(-0.009,0.055)	0.0318	100.3	-1.45
C5.OH(C3.DC.M)	**Val**	0(0,0)	0.01567	323.7	-2.07
C5	**Leu**	0(0,0)	0.01108	97.2	1.97
PCaa40:6	**PCae38:0**	2.749(0.158,5.34)	0.00541	971.5	-2.82
PCaa38:6	**PCae38:0**	6.81(-0.652,14.272)	0.01075	212.3	-1.75
SM(OH)22:1	**SM18:1**	0.719(0.133,1.305)	0.00293	645.5	-2.3
SM(OH)22:1	**SM16:0**	0.027(0.006,0.049)	0.00293	606.9	-2.46
SM(OH)16:1	**SM(OH)22:1**	-0.047(-0.091,-0.003)	0.00395	123.1	2.99
PCaa36:5	**SM24:0**	-0.139(-0.245,-0.033)	0.00286	13603.3	3.29
PCaa36:2	**SM24:0**	-7.469(-13.593,-1.346)	0.00352	11205.3	2.75
PCae38:0	**SM16:1**	-0.086(-0.155,-0.017)	0.00293	10982.2	3.93
PCae40:4	**SM24:0**	-0.043(-0.074,-0.012)	0.00286	9723.6	2.83
PCae42:4	**SM24:0**	-0.01(-0.016,-0.004)	0.00107	9595.6	3.62
PCae40:5	**SM24:0**	-0.02(-0.036,-0.003)	0.00352	8631.1	2.68
PCae40:2	**SM24:0**	-0.012(-0.023,-0.002)	0.00352	7987.9	2.79
PCae40:1	**SM(OH)22:1**	-0.645(-1.086,-0.203)	0.00107	7524.5	4.12
PCaa36:5	**SM(OH)22:2**	-1.27(-2.327,-0.214)	0.00293	7120.5	3.49
PCaa34:2	**SM24:0**	-7.637(-14.187,-1.088)	0.00356	6942.4	2.61
PCae38:3	**SM24:0**	-0.026(-0.048,-0.005)	0.00352	5987.3	2.71
PCae38:0	**SM(OH)22:1**	-0.422(-0.719,-0.126)	0.00175	5679.5	4.22
PCae38:0	**SM(OH)22:2**	-0.611(-1.107,-0.116)	0.00346	5503.7	2.68
PCae40:1	**SM(OH)22:2**	-0.948(-1.727,-0.17)	0.00352	5229.5	3.09
PCaa36:5	**SM(OH)22:1**	-0.873(-1.552,-0.194)	0.00175	4815.3	4.77
PCae40:1	**SM24:0**	-0.104(-0.195,-0.014)	0.00352	4336.7	2.67
PCae38:5	**SM24:0**	-0.07(-0.131,-0.009)	0.00464	3588.2	2.35
PCae38:0	**SM24:0**	-0.069(-0.129,-0.008)	0.00353	2873.8	3.59
PCae36:3	**SM24:0**	-0.023(-0.045,-0.001)	0.0053	2712.1	2.2
PCaa36:5	**SM16:1**	-0.176(-0.325,-0.027)	0.00352	2480.1	3
PCae38:5	**SM16:1**	-0.084(-0.164,-0.004)	0.00648	2179.1	2.21
PCae38:4	**SM(OH)22:1**	-0.694(-1.196,-0.192)	0.00284	2109.5	3.46
PCaa32:2	**SM24:0**	-0.025(-0.047,-0.003)	0.00293	2094.7	4.04
PCaa36:6	**SM24:0**	-0.009(-0.018,-0.001)	0.00527	1873	2.63
PCae38:6	**SM16:1**	-0.059(-0.12,0.002)	0.00723	1705.8	2.57
PCae38:4	**SM(OH)22:2**	-0.963(-1.885,-0.041)	0.00594	1594.2	2.14
PCaa36:4	**SM(OH)22:1**	-35.398(-63.36,-7.437)	0.00284	1582.2	3.58
PCaa34:4	**SM24:0**	-0.018(-0.036,-0.001)	0.00665	1435.8	2.2
PCae38:5	**SM(OH)22:2**	-0.65(-1.266,-0.033)	0.00633	1366.2	1.94
PCae38:4	**SM16:1**	-0.13(-0.264,0.004)	0.00723	1353.8	2.76
PCaa36:3	**SM24:0**	-2.735(-5.279,-0.192)	0.0053	1345.5	2.13
PCae36:5	**SM(OH)22:1**	-0.283(-0.5,-0.066)	0.00286	1317.5	3.7
PCae40:5	**SM16:1**	-0.023(-0.046,0.001)	0.00847	1177	1.85

PCae40:5	**SM(OH)22:2**	-0.187(-0.369,-0.004)	0.00695	1141.8	2.09
PCae38:4	**SM24:0**	-0.106(-0.214,0.002)	0.00801	1080.7	2.62
PCaa36:4	**SM16:1**	-6.247(-12.989,0.494)	0.01011	1044	2.45
PCaa38:5	**SM(OH)22:1**	-5.849(-10.639,-1.06)	0.00293	1012.6	3.82
PCae40:4	**SM16:1**	-0.054(-0.102,-0.007)	0.00401	978.9	2.47
PCae38:6	**SM24:0**	-0.048(-0.098,0.001)	0.00847	899.3	2.54
PCae36:4	**SM(OH)22:2**	-0.771(-1.582,0.04)	0.0077	860.6	1.82
PCae36:4	**SM(OH)22:1**	-0.548(-0.97,-0.126)	0.00293	822.3	3.06
PCae36:4	**SM24:0**	-0.086(-0.175,0.003)	0.00815	817.5	2.27
PCaa42:4	**SM24:0**	-0.006(-0.012,0)	0.00759	804.3	2.55
PCaa30:0	**SM24:0**	-0.014(-0.029,0)	0.00732	734.5	2.7
PCae40:1	**SM16:1**	-0.133(-0.27,0.005)	0.00678	668.4	2.47
PCae38:6	**SM(OH)22:2**	-0.444(-0.902,0.014)	0.00848	639	2.07
PCae40:3	**SM24:0**	-0.013(-0.028,0.001)	0.01053	595.1	2.13
PCae34:2	**SM24:0**	-0.052(-0.109,0.005)	0.01108	529.6	2.05
PCae44:4	**SM24:0**	-0.004(-0.008,-0.001)	0.00293	478.2	3.08
PCae40:4	**SM(OH)22:2**	-0.389(-0.765,-0.014)	0.00596	453.8	2.04
PCae42:4	**SM(OH)22:1**	-0.056(-0.102,-0.01)	0.00284	446.2	3.76
PCaa34:2	**SM(OH)22:1**	-56.33(-105.84,-6.827)	0.00346	445.8	3.45
PCae36:4	**SM16:1**	-0.107(-0.228,0.013)	0.01108	434.9	2.32
PCae42:2	**SM24:0**	-0.011(-0.023,0.001)	0.01168	417.7	2.46
PCae38:6	**SM(OH)22:1**	-0.326(-0.595,-0.057)	0.00346	410.7	3.43
PCaa36:3	**SM16:1**	-3.481(-7.116,0.153)	0.00943	382.8	2.18
PCaa34:2	**SM16:1**	-9.162(-19.734,1.409)	0.01504	379.4	2.58
PCaa34:3	**SM24:0**	-0.224(-0.478,0.031)	0.01279	378	2.03
PCaa36:2	**SM(OH)22:1**	-54.08(-101.38,-6.774)	0.00352	373.1	3.41
PCaa40:4	**SM24:0**	-0.07(-0.151,0.011)	0.01212	371.2	2.09
PCaa36:6	**SM(OH)22:2**	-0.084(-0.176,0.008)	0.01108	351.6	2.15
PCaa36:2	**SM16:1**	-9.053(-19.44,1.333)	0.01364	351.6	2.77
PCae38:5	**SM(OH)22:1**	-0.493(-0.905,-0.081)	0.00352	347.5	3.26
PCaa34:1	**SM24:0**	-3.56(-7.535,0.414)	0.0071	347.2	2.99
PCae44:5	**SM24:0**	-0.003(-0.006,0)	0.00807	322.5	2.03
PCaa38:4	**SM(OH)22:1**	-33.003(-61.44,-4.568)	0.00352	322.2	2.94
PCaa36:4	**SM(OH)22:2**	-47.508(-102.44,7.423)	0.01212	318.5	1.8
PCaa36:6	**SM16:1**	-0.011(-0.023,0.001)	0.01168	315.6	2.03
PCaa40:5	**SM(OH)22:1**	-1.269(-2.464,-0.074)	0.00352	283.5	3.64
PCaa36:4	**SM24:0**	-5.163(-11.107,0.781)	0.01531	283.4	2.22
PCaa42:4	**SM16:1**	-0.007(-0.015,0.001)	0.01236	273.5	1.91
PCae38:0	**SM16:0**	-0.015(-0.032,0.001)	0.00988	264.5	2.62
PCae40:4	**SM(OH)22:1**	-0.277(-0.525,-0.03)	0.00352	255	3.3
PCaa36:5	**SM16:0**	-0.03(-0.061,0.001)	0.00847	253	2.12
PCae40:6	**SM(OH)22:1**	-0.244(-0.453,-0.036)	0.00352	247.8	2.93
PCae40:6	**SM24:0**	-0.03(-0.065,0.005)	0.01626	245.9	2
PCae34:3	**SM24:0**	-0.024(-0.047,-0.002)	0.00594	243	2.67
PCae34:1	**SM16:1**	-0.029(-0.067,0.008)	0.02871	235.8	1.68
PCae36:1	**SM24:0**	-0.045(-0.099,0.009)	0.01212	232.9	2.6
PCae40:6	**SM(OH)22:2**	-0.293(-0.63,0.044)	0.01462	230.3	1.66

PCae42:3	**SM24:0**	-0.029(-0.055,-0.003)	0.00388	223.6	2.24
PCaa32:2	**SM(OH)22:1**	-0.153(-0.311,0.005)	0.00352	222.9	5.28
PCae36:5	**SM(OH)22:2**	-0.388(-0.87,0.093)	0.01504	222.3	2.21
PCaa34:3	**SM(OH)22:1**	-1.716(-3.392,-0.041)	0.00356	201.4	3.56
PCaa32:0	**SM24:0**	-0.15(-0.339,0.039)	0.01821	199.7	2.87
PCaa36:2	**SM(OH)22:2**	-70.24(-153.82,13.347)	0.01568	194.9	1.76
PCae40:5	**SM(OH)22:1**	-0.148(-0.285,-0.011)	0.00356	193.7	3.35
PCae36:3	**SM(OH)22:1**	-0.17(-0.331,-0.009)	0.00356	179.9	3.62
PCaa38:5	**SM(OH)22:2**	-7.451(-16.166,1.265)	0.0163	179.8	1.85
PCaa36:6	**SM(OH)22:1**	-0.062(-0.122,-0.002)	0.00356	178.1	3.5
PCae44:4	**SM(OH)22:1**	-0.024(-0.05,0.001)	0.00352	177.4	3.91
PCaa34:4	**SM(OH)22:1**	-0.124(-0.237,-0.01)	0.00356	177.2	3.38
PCaa34:2	**SM(OH)22:2**	-72.10(-160.59,16.382)	0.01647	173.6	1.67
PCae40:2	**SM(OH)22:2**	-0.118(-0.26,0.023)	0.01567	173.3	2.16
PCaa36:3	**SM(OH)22:1**	-17.601(-34.15,-1.051)	0.00356	172	3.19
PCae44:6	**SM24:0**	-0.003(-0.006,0)	0.01108	171	1.67
PCae40:1	**SM16:0**	-0.023(-0.049,0.003)	0.01241	167	1.88
PCae34:3	**SM(OH)22:1**	-0.147(-0.28,-0.013)	0.00365	161.8	3.94
PCaa38:5	**SM16:1**	-0.918(-2.171,0.336)	0.03239	159.2	1.84
PCaa34:1	**SM(OH)22:1**	-21.275(-45.159,2.609)	0.00365	158.2	4.41
PCae42:3	**SM(OH)22:1**	-0.166(-0.325,-0.007)	0.00366	156.1	3.21
PCae44:4	**SM16:1**	-0.006(-0.011,-0.001)	0.00356	155	2.38
PCae34:1	**SM24:0**	-0.026(-0.058,0.005)	0.0212	154.5	1.89
PCae40:2	**SM(OH)22:1**	-0.09(-0.182,0.001)	0.00373	147.1	4.07
PCaa32:1	**SM(OH)22:1**	-0.425(-0.868,0.019)	0.00352	144.2	5
PCae36:3	**SM(OH)22:2**	-0.221(-0.497,0.055)	0.01886	140	1.89
PCae36:3	**SM16:1**	-0.028(-0.066,0.01)	0.02847	134.4	2.04
PCaa34:4	**SM(OH)22:2**	-0.169(-0.375,0.037)	0.01936	133.5	1.83
PCaa36:3	**SM(OH)22:2**	-24.923(-55.12,5.274)	0.01568	132.5	1.77
PCae42:4	**SM16:1**	-0.013(-0.024,-0.002)	0.00346	128.3	2.61
PCaa38:6	**SM(OH)22:1**	-16.04(-31.631,-0.445)	0.00395	124.8	3.43
PCae38:3	**SM16:1**	-0.032(-0.07,0.006)	0.01798	123.1	2.16
PCae38:4	**SM16:0**	-0.022(-0.05,0.006)	0.02499	117.5	1.72
PCaa40:4	**SM16:1**	-0.086(-0.198,0.027)	0.02322	115	1.82
PCae40:6	**SM16:1**	-0.034(-0.085,0.018)	0.04101	113.3	1.67
PCaa34:2	**SM16:0**	-1.477(-3.408,0.455)	0.02838	108.7	1.5
PCaa32:1	**SM(OH)22:2**	-0.684(-1.379,0.011)	0.00356	106.7	3.86
PCaa32:1	**SM16:1**	-0.104(-0.201,-0.007)	0.00356	105.7	3.57
PCaa40:2	**SM24:0**	-0.015(-0.034,0.005)	0.0218	105.4	2.21
PCaa36:2	**SM16:0**	-1.473(-3.363,0.417)	0.02636	103.7	1.61
PCaa32:2	**SM(OH)22:2**	-0.23(-0.485,0.026)	0.00813	102.1	3.38
PCae42:5	**SM24:0**	-0.01(-0.021,0.001)	0.01164	101.9	1.84
PCaa34:3	**SM(OH)22:2**	-2.166(-5.053,0.722)	0.02263	101.6	1.76
PCaa34:1	**SM(OH)22:2**	-32.111(-73.922,9.701)	0.01241	99.6	2.64
C16(OH)	**SM(OH)22:1**	-0.002(-0.003,0)	0.00293	735.3	3.81
C18:1(OH)	**SM(OH)22:1**	-0.002(-0.004,0)	0.00352	220.1	2.95
C4	**SM(OH)22:1**	-0.084(-0.157,-0.011)	0.00352	217.4	2.64

SGLT2-Inhibitor

SGLT2-Inhibitor testis 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

SGLT2-Inhibitor adrenal gland 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

SGLT2-Inhibitor lung 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

SGLT2-Inhibitor cerebellum 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

SGLT2-Inhibitor plasma 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
		No significant changes			

Combination treatment

Combination treatment testis 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		428.0(277.06,578.95)	1.67E-07		-5.81
lysoPC18:2		-0.954(-1.851,-0.057)	0.00228		2.67
Lys		-8.33(-19.509,2.849)	0.03531		2.37
C5.OH(C3.DC.M)	**Val**	0.002(-0.001,0.005)	0.08006	225.5	-1.54

C5.OH(C3.DC.M)	**Leu**	0.003(-0.002,0.008)	0.0696	116.7	-1.24
Gln	**Pro**	0.885(0.012,1.758)	0.0046	274.9	-2.34
Lys	**Trp**	-2.738(-4.858,-0.618)	0.00045	184.2	2.82
Lys	**Phe**	-0.525(-0.978,-0.072)	0.00104	75.6	3.33
SM(OH)14:1	**SM16:0**	-0.001(-0.002,0)	0.03254	128.4	1.58
C5.OH(C3.DC.M)	**lysoPC14:0**	0.106(-0.06,0.271)	0.07138	187.6	-1.3
Creatinine	**lysoPC18:2**	16.557(5.544,27.57)	1.67E-07	382.1	-2.48
PCae34:1	**Putrescine**	0.794(0.051,1.536)	0.00205	378.2	-1.95
Putrescine	**SM18:1**	-6.308(-12.123,-0.493)	0.00199	388.7	3.23
PCaa28:1	**Putrescine**	0.016(0.001,0.031)	0.00324	225.1	-3.19
Putrescine	**SM16:0**	-0.019(-0.038,-0.001)	0.00398	175	2.17
PCaa32:0	**Putrescine**	0.529(-0.037,1.095)	0.00554	114.2	-1.56
PCaa40:4	**Putrescine**	0.104(-0.001,0.209)	0.00727	79.4	-3.24
PCaa40:6	**Putrescine**	0.51(-0.034,1.055)	0.00877	62.4	-2.22

Combination treatment adrenal gland 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
C5.OH(C3.DC.M)	**PCae38:0**	0.018(0.003,0.033)	0.0964	23375.1	-2.62
C5.OH(C3.DC.M)	**PCaa38:0**	0.023(0,0.045)	0.0964	6048.5	-1.92
C5.OH(C3.DC.M)	**Lys**	0.002(0,0.003)	0.13317	5876.4	-2.15
H1	**His**	59.54(12.52,106.56)	0.03481	4536.3	-2.46
PCaa32:3	**PCaa40:6**	0.004(0,0.007)	0.0964	3051.5	-1.85
C5	**Orn**	0.018(0,0.035)	0.13317	422.4	-1.9
Sum of hexoses	**PCaa42:1**	8283.2(479.73,16086)	0.0964	366.6	-2.45
C5.OH(C3.DC.M)	**Pro**	0.001(0,0.003)	0.17293	252.8	-1.42

Combination treatment lung 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		1613(882.5,2343)	1.30E-07		-3.93
C16:1(OH)		-0.05(-0.095,-0.004)	0.00102		3.28
C7(DC)		-0.018(-0.035,0)	0.00192		3.29
C14:1(OH)		-0.024(-0.048,0)	0.00225		2.54
C16(OH)		-0.123(-0.254,0.007)	0.00261		2.9
C18:1(OH)		-0.137(-0.315,0.041)	0.01135		2.33
C16:2(OH)		-0.011(-0.029,0.006)	0.02509		1.94
C2		-12.336(-30.673,6.001)	0.02681		1.74
C14:2(OH)		-0.008(-0.021,0.004)	0.03016		1.72
C12:1		-0.016(-0.044,0.011)	0.0303		1.13
C5-DC(C6-OH)		-0.013(-0.034,0.008)	0.03325		1.59
C5:1		-0.003(-0.009,0.002)	0.03349		1.19
C16:1		-0.073(-0.209,0.064)	0.03879		1.89
C14:1		-0.029(-0.082,0.025)	0.04294		1.9
Lys		-10.28(-23.658,3.098)	0.01032		2.49

Met		-2.66(-7.181,1.861)	0.0372		1.26
PCaa36:3		8.674(-4.025,21.374)	0.02004		-1.46
PCaa34:2		52.05(-27.34,131.44)	0.02624		-1.43
PCaa34:3		2.105(-1.628,5.839)	0.04555		-1.08
lysoPC18:2		-6.571(-14.04,0.897)	0.00456		2.89
lysoPC18:1		-1.466(-3.626,0.695)	0.01993		1.43
lysoPC16:0		-6.387(-17.185,4.412)	0.0353		1.12
lysoPC17:0		-0.058(-0.163,0.047)	0.04628		1.22
lysoPC20:4		-0.875(-2.569,0.819)	0.04978		1.04
SM16:1		0.885(-0.551,2.321)	0.03286		-1.26
C5-DC(C6-OH)	**C5.OH(C3.DC.M)**	-0.097(-0.169,-0.025)	4.00E-05	37982.2	6.61
C14:2(OH)	**C4:1**	-1.212(-2.212,-0.212)	0.00037	914	4.32
C3-DC (C4-OH)	**C4:1**	-43.151(-84.26,-2.041)	0.00148	777.3	3.2
C2	**C5.OH(C3.DC.M)**	-83.38(-152.5,-14.24)	0.00037	670.3	5.39
C14:2(OH)	**C5.OH(C3.DC.M)**	-0.058(-0.108,-0.008)	0.00058	468.2	3.29
C18:1(OH)	**C5.OH(C3.DC.M)**	-0.833(-1.488,-0.177)	0.00021	279.4	3.53
C16(OH)	**C5.OH(C3.DC.M)**	-0.723(-1.202,-0.244)	1.30E-07	213.2	3.7
C2	**C4:1**	-1704(-3420,11.077)	0.001	193	4.13
C16:1	**C18:1**	-0.034(-0.066,-0.002)	0.00219	142.8	2.17
C5-DC(C6-OH)	**C8**	-0.134(-0.329,0.061)	0.02245	122.9	2.05
C6(C4:1(DC))	**C8**	-0.236(-0.604,0.132)	0.02282	119.5	2.22
C5.OH(C3.DC.M)	**C7(DC)**	2.91(0.794,5.027)	1.30E-07	116.2	-2.47
C5.OH(C3.DC.M)	**C6(C4:1(DC))**	0.61(-0.029,1.249)	0.00414	98	-2.07
C16:2(OH)	**C5.OH(C3.DC.M)**	-0.076(-0.142,-0.009)	0.00171	89.2	2.5
C4:1	**Ile**	0(0,0)	0.00358	226.1	-2.11
C5.OH(C3.DC.M)	**Val**	0.001(0,0.002)	0.01242	179.6	-1.81
C5-DC(C6-OH)	**Gly**	0(0,0)	0.02196	167.6	1.86
C5-DC(C6-OH)	**Gln**	0(0,0)	0.02318	164.2	2.07
C5.OH(C3.DC.M)	**Ile**	0.002(-0.001,0.005)	0.0136	83.6	-1.46
PCaa38:3	**PCae38:4**	0.66(0.2,1.119)	4.00E-05	24358.3	-2.86
PCaa38:3	**PCae32:2**	0.851(0.195,1.507)	0.00028	6424.2	-2.64
PCaa34:1	**PCae32:2**	10.439(0.877,20.001)	0.00102	4328.7	-2.09
PCaa32:2	**PCaa32:3**	0.811(0.164,1.458)	0.00064	4192.4	-3.12
PCaa38:3	**PCae36:5**	0.14(0.018,0.261)	0.00083	1432.8	-2.19
PCaa34:2	**PCaa36:4**	0.259(0.058,0.46)	0.00021	1235.7	-2.29
PCaa32:3	**PCaa34:3**	-0.082(-0.157,-0.006)	0.00052	1175.3	5.05
PCaa38:3	**PCaa40:4**	0.42(0.032,0.809)	0.00119	913.4	-1.99
PCaa34:1	**PCae38:4**	8.663(-0.363,17.69)	0.00336	891.5	-1.93
PCaa34:2	**PCae36:5**	3.316(0.635,5.998)	0.00037	726.1	-2.31
PCaa38:3	**PCaa38:4**	0.023(0.001,0.045)	0.0021	444.9	-2.18
PCaa38:3	**PCaa42:5**	5.968(0.065,11.871)	0.00225	404.3	-2.1
PCaa38:3	**PCaa42:6**	3.34(0.156,6.524)	0.00232	386.6	-2.17
PCaa38:3	**PCae40:4**	2.618(-0.04,5.276)	0.00246	359.5	-1.66
PCaa38:3	**PCae40:5**	1.586(0.015,3.156)	0.00258	340.3	-1.87
PCaa36:2	**PCae36:5**	1.075(-0.001,2.151)	0.00208	332.6	-1.68
PCaa32:3	**PCae42:2**	-3.949(-8.241,0.343)	0.00429	331.1	2.28
PCaa38:3	**PCae38:5**	0.249(0,0.498)	0.0028	296.8	-1.86

PCaa34:3	PCaa34:4	1.158(0.135,2.181)	0.00169	281.2	-3.29
PCaa36:3	PCae36:5	0.557(0.091,1.022)	0.00049	276.8	-2.06
PCaa36:3	PCae38:6	1.385(0.212,2.559)	0.00049	269.2	-2.01
PCaa36:2	PCae38:4	5.106(-0.079,10.292)	0.00258	251.4	-1.67
PCaa38:3	PCaa40:5	0.371(-0.009,0.751)	0.00306	250.1	-1.78
PCae34:2	PCae38:4	0.326(-0.079,0.732)	0.00792	227.5	-1.39
PCaa40:2	PCae38:4	0.017(-0.004,0.038)	0.00755	226.6	-1.91
PCaa40:3	PCae40:4	0.102(-0.011,0.214)	0.00595	223.6	-2.52
PCaa36:3	PCaa36:4	0.043(0.006,0.08)	6.00E-04	212.6	-2.19
PCaa40:2	PCae40:4	0.065(-0.015,0.145)	0.00789	207.9	-1.84
PCaa36:2	PCaa38:4	0.179(-0.009,0.368)	0.003	196.3	-1.56
PCaa36:2	PCae32:2	6.543(-0.351,13.437)	0.00301	191.2	-1.68
PCaa38:3	PCae36:4	0.233(-0.012,0.479)	0.00371	190.9	-1.9
PCaa38:3	PCae40:6	1.818(-0.062,3.699)	0.00406	160.9	-1.85
PCae36:3	PCae36:4	0.052(-0.004,0.108)	0.00414	156.6	-1.83
PCaa32:1	PCae36:5	2.158(-0.455,4.771)	0.01	146.8	-2.06
PCaa34:2	PCae38:6	8.335(0.484,16.187)	0.00124	139.7	-1.84
PCaa34:3	PCae38:0	1.311(0.005,2.618)	0.00281	137.9	-2.19
PCae32:2	PCae34:2	-0.037(-0.083,0.009)	0.01076	136.6	1.83
PCaa32:2	PCae38:0	1.606(-0.245,3.457)	0.00694	136.1	-2.11
PCae34:1	PCae38:4	0.311(-0.122,0.744)	0.01759	120.2	-1.5
PCaa40:3	PCae38:4	0.026(-0.005,0.058)	0.00857	120	-2.36
PCaa34:2	PCae36:4	5.6(0.065,11.135)	0.00144	117.5	-1.7
PCae36:3	PCae42:1	0.809(-0.084,1.702)	0.00474	117.1	-1.6
PCaa34:3	PCaa36:6	4.806(-0.106,9.717)	0.00312	114.1	-1.85
PCae36:3	PCae38:6	0.077(-0.013,0.167)	0.00494	110	-1.54
PCae36:3	PCae38:5	0.055(-0.01,0.121)	0.00551	93.1	-1.46
PCae36:5	SM18:1	-2.274(-4.71,0.162)	0.00405	187.7	1.94
lysoPC17:0	PCaa34:3	-0.006(-0.01,-0.001)	6.00E-04	982.2	3
lysoPC16:0	SM16:1	-1.802(-3.548,-0.056)	0.00194	122.3	2.17
C5.OH(C3.DC.M)	lysoPC18:1	0.013(0.003,0.023)	0.00037	408.1	-2.9
C5.OH(C3.DC.M)	lysoPC18:2	0.006(0.002,0.01)	4.00E-05	228.3	-3.39
C5.OH(C3.DC.M)	lysoPC20:4	0.017(0,0.034)	0.00335	122.5	-2.33

Combination treatment cerebellum 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		43.072(5.938,80.207)	0.00017		-1.91
C0		-5.119(-11.124,0.886)	0.01379		1.93
Carnosine		-2.138(-5.391,1.115)	0.04189		1.34
PCae38:0		-0.341(-0.703,0.022)	0.00975		1.8
PCaa36:6		-0.052(-0.109,0.004)	0.01072		1.63
PCae42:3		-0.425(-0.905,0.055)	0.01465		1.54
PCae44:5		-0.833(-1.857,0.192)	0.02169		1.51
PCae44:4		-0.06(-0.135,0.015)	0.02276		1.7
PCae44:3		-0.104(-0.238,0.031)	0.02523		1.54

PCaa36:5		-0.215(-0.518,0.087)	0.03461		1.29
PCaa38:5		-3.15(-7.6,1.3)	0.03895		1.25
lysoPC20:4		-0.75(-1.597,0.097)	0.01436		2.11
lysoPC18:2		-0.167(-0.367,0.034)	0.01503		1.77
lysoPC16:0		-3.33(-7.379,0.72)	0.01836		1.69
lysoPC16:1		-0.091(-0.213,0.032)	0.033		1.53
lysoPC18:1		-1.86(-4.524,0.804)	0.03841		1.58
Lys	**Trp**	-0.836(-1.654,-0.019)	0.00419	275.9	2.23
Carnosine	**Trp**	-0.97(-1.803,-0.136)	0.00092	251.7	2.7
Phe	**Trp**	-0.382(-0.946,0.183)	0.03748	121.6	2.38
C2	**C3**	-3.698(-7.082,-0.315)	0.00332	747.9	2.17
C18:1	**C18:2**	-1.503(-3.215,0.209)	0.01653	173.5	2.05
C3	**C5.OH(C3.DC.M)**	1.397(-0.278,3.071)	0.01194	130.6	-1.52
PCaa34:2	**PCae42:3**	5.915(2.791,9.039)	8.00E-05	40503.7	-3.54
PCaa34:2	**PCaa38:5**	0.455(0.165,0.746)	8.00E-05	13315.4	-2.84
PCaa34:2	**PCaa36:4**	0.111(0.023,0.198)	0.00042	5510.8	-2.39
PCaa34:2	**PCaa36:5**	9.581(2.977,16.185)	0.00017	3679.4	-2.61
PCaa34:2	**PCae42:0**	2.897(0.544,5.249)	0.00037	1640.9	-2.13
PCaa34:2	**PCae44:5**	3.33(0.847,5.813)	0.00017	1031.9	-2.38
PCae40:1	**PCae42:3**	0.307(0.088,0.526)	0.00017	471.9	-2.63
PCaa34:3	**PCae36:3**	0.133(-0.028,0.294)	0.01743	461.7	-1.61
PCaa34:2	**PCae44:3**	19.433(4.027,34.838)	0.00029	377.9	-2.37
PCaa34:2	**PCaa38:6**	0.077(0,0.155)	0.00441	301.3	-1.8
PCaa34:2	**PCaa40:5**	2.292(0.241,4.343)	0.00137	257.7	-1.98
PCae34:2	**PCae38:2**	0.237(-0.018,0.492)	0.00846	202.7	-2.06
PCaa34:2	**PCae36:3**	8.37(-0.215,16.955)	0.00609	198.4	-2.18
PCaa34:2	**PCaa38:0**	13.435(-0.504,27.373)	0.00515	192.5	-1.69
PCaa34:3	**PCae44:5**	0.075(0.012,0.137)	0.00042	190.3	-2.05
PCaa34:2	**PCaa36:2**	0.124(-0.004,0.253)	0.00637	185.1	-1.95
PCaa38:3	**PCaa40:5**	0.124(0.008,0.24)	0.00187	182.3	-2.03
PCae40:1	**PCae44:5**	0.176(0.029,0.323)	0.00051	147.8	-2.06
PCaa34:2	**PCaa34:4**	53.336(-1.529,108.2)	0.00515	129.7	-2.24
PCaa34:3	**PCae38:5**	0.246(-0.057,0.549)	0.01836	116.9	-1.63
PCaa34:3	**PCae42:3**	0.13(0.031,0.228)	0.00037	116.6	-3.04
PCaa34:2	**PCae38:4**	8.996(-0.909,18.902)	0.00964	106.5	-1.62
PCaa36:3	**PCae36:3**	1.702(-0.842,4.246)	0.03985	101.9	-1.13
PCaa34:3	**PCaa38:5**	0.009(0.001,0.017)	0.00177	99.6	-2.36
PCaa36:3	**PCaa38:5**	0.112(0.006,0.217)	0.00177	99.3	-1.81
Histamine	**PCaa36:2**	0(0,0.001)	0.00491	732.1	-1.94
Histamine	**PCaa32:1**	0.002(0,0.004)	0.00053	721.2	-2.21
Histamine	**PCae42:2**	0.049(0.002,0.096)	0.00378	295.1	-1.91
Histamine	**PCae38:5**	0.044(-0.006,0.093)	0.01131	258.5	-1.48
Histamine	**PCae44:5**	0.013(0.002,0.023)	0.00051	155.5	-2.03
lysoPC18:1	**Trp**	-0.808(-1.327,-0.289)	8.00E-05	8311.1	3.91
lysoPC16:0	**Trp**	-1.459(-2.35,-0.568)	0.00017	1433	3.36
Arg	**lysoPC18:1**	0.579(0.099,1.06)	0.00029	792.2	-2.09
lysoPC16:1	**Trp**	-0.039(-0.068,-0.01)	6.00E-04	239	2.87

lysoPC20:4	Trp	-0.318(-0.54,-0.095)	0.00023	237.1	3.48

Combination treatment plasma 14d

Metabolite 1	Metabolite 2	95% CI	q-value	p-gain	effect size
Sum of hexoses		16460(8056,24864)	1.56E-06		-4.55
C4:1		0.037(0.011,0.062)	2.00E-05		-2.89
C10		0.091(0.009,0.173)	0.00022		-2.32
C12:1		0.012(-0.007,0.03)	0.01083		-1.88
C18		0.015(-0.011,0.041)	0.01918		-1.22
Cit		-15.54(-36.1,5.02)	0.00476		2.55
Lys		-62.1(-164.952,40.752)	0.01692		1.49
Pro		-20.62(-62.972,21.732)	0.03686		1.26
alphaAAA		-1.724(-4.129,0.681)	0.00522		1.82
PCae38:2		-2.922(-6.021,0.177)	0.00048		3.28
PCae38:1		-0.5(-1.225,0.226)	0.00557		2.22
PCae42:4		-0.152(-0.403,0.1)	0.00744		4.31
PCae44:4		-0.048(-0.123,0.027)	0.00867		2.69
PCae36:0		-0.09(-0.229,0.05)	0.01139		1.61
PCae36:2		-2.633(-7.23,1.964)	0.0135		1.65
PCae34:2		-0.801(-2.096,0.494)	0.01402		1.54
PCae40:4		-0.674(-1.829,0.481)	0.01464		2.24
PCae34:3		-0.294(-0.753,0.165)	0.01677		2.43
PCae42:3		-0.472(-1.417,0.473)	0.0197		2.32
PCae40:2		-0.195(-0.567,0.177)	0.02146		2.62
PCae42:5		-0.123(-0.349,0.103)	0.02271		2.88
PCaa36:1		-19.03(-54.345,16.285)	0.02342		1.42
PCae34:0		-0.052(-0.143,0.039)	0.02544		1.3
PCae36:1		-0.499(-1.487,0.489)	0.02711		1.7
PCaa42:2		-0.077(-0.246,0.092)	0.02748		1.78
PCae44:6		-0.037(-0.106,0.032)	0.02755		1.57
PCae38:6		-0.755(-2.202,0.692)	0.03103		1.66
PCaa32:0		-1.766(-5.295,1.763)	0.03747		1.22
PCae36:4		-0.976(-2.913,0.961)	0.03788		1.29
PCaa40:2		-0.188(-0.616,0.239)	0.04492		2.55
lysoPC18:1		-9.38(-26.075,7.315)	0.02392		1.19
lysoPC18:2		-51.2(-161.504,59.104)	0.04609		0.99
SM(OH)22:1		1.414(-0.541,3.369)	0.00669		-1.45
alphaAAA	Ser	-0.02(-0.034,-0.006)	2.00E-05	2240.2	6.13
Lys	Ser	-0.752(-1.497,-0.007)	1.00E-04	1083.1	7.39
alphaAAA	Trp	-0.021(-0.035,-0.006)	5.00E-05	443.7	3.19
C10	C8	0.867(0.383,1.351)	1.56E-06	188	-4.17
alphaAAA	C12:1	-61.34(-93.75,-28.94)	5.00E-05	17636.2	4.68
C12:1	Lys	0(0,0)	8.00E-05	908.9	-2.48
alphaAAA	C10	-15.93(-26.31,-5.555)	8.00E-05	210	5.28
alphaAAA	C12	-41.76(-75.56,-7.952)	0.00015	104.2	2.93

alphaAAA	**C5.OH(C3.DC.M)**	-47.652(-88.34,-6.964)	0.00016	98.8	3.23
PCaa38:3	**PCaa40:4**	4.857(2.477,7.237)	1.56E-06	5944323.8	-3.86
PCaa40:6	**PCae38:0**	3.901(1.595,6.207)	1.56E-06	1272230.7	-4.01
PCaa40:6	**PCae40:6**	4.258(1.303,7.213)	2.00E-05	302652.8	-2.58
PCaa38:3	**PCae38:6**	8.686(3.84,13.531)	1.56E-06	271428.9	-3.57
PCaa40:6	**PCae38:4**	2.204(0.791,3.617)	1.56E-06	215406.1	-3.38
PCaa38:3	**PCae38:4**	3.539(1.445,5.634)	1.56E-06	170747.7	-3.43
PCaa38:3	**PCae34:1**	8.673(3.088,14.258)	1.56E-06	93482	-2.94
PCaa38:3	**PCae40:4**	11.727(5.444,18.01)	1.56E-06	92591.4	-3.64
PCaa38:3	**PCae38:5**	4.795(1.805,7.785)	1.56E-06	89482.7	-3.38
PCaa38:3	**PCae36:4**	5.042(2.02,8.065)	1.56E-06	79958	-3.51
PCaa38:3	**PCae42:0**	30.775(10.045,51.505)	1.56E-06	73071.1	-2.64
PCaa34:1	**PCaa34:3**	-2.23(-3.713,-0.747)	2.00E-05	49183.6	3.4
PCaa32:0	**PCaa38:3**	-0.07(-0.118,-0.023)	2.00E-05	36615.8	4.17
PCaa38:4	**PCae38:4**	8.617(2.072,15.163)	8.00E-05	26834.5	-2.68
PCaa38:3	**PCae38:0**	6.306(2.026,10.585)	2.00E-05	24076.6	-3.34
PCaa40:6	**PCae40:4**	8.907(3.096,14.718)	2.00E-05	12098	-2.99
PCaa38:3	**PCaa42:5**	54.089(12.164,96.015)	5.00E-05	11155.5	-2.34
PCaa38:3	**PCae40:1**	4.563(1.103,8.022)	5.00E-05	9990	-2.58
PCaa38:3	**PCae42:3**	27.527(9.296,45.758)	2.00E-05	9235.4	-2.97
PCaa40:6	**PCae40:1**	3.097(0.572,5.621)	0.00015	8290.1	-2.46
PCaa38:3	**PCae38:3**	12.357(2.281,22.433)	8.00E-05	6857.9	-2.19
PCaa36:4	**PCaa38:3**	-1.671(-3.04,-0.303)	8.00E-05	6679.9	3.34
PCaa36:3	**PCaa38:3**	-0.796(-1.425,-0.167)	8.00E-05	6411.2	3.22
PCaa38:3	**PCae40:5**	15.473(2.861,28.084)	8.00E-05	6374.6	-2.18
PCaa34:1	**PCaa38:3**	-1.057(-1.897,-0.216)	8.00E-05	6151.9	3.26
PCaa38:3	**PCae34:2**	8.075(2.494,13.657)	2.00E-05	5485	-2.62
PCaa38:3	**PCae36:3**	13.227(2.344,24.111)	1.00E-04	5295.3	-2.13
PCaa38:3	**PCaa42:4**	66.30(11.056,121.6)	0.00012	5063	-2.21
PCaa40:6	**PCae38:6**	6.288(1.617,10.959)	8.00E-05	3544.3	-3.09
PCaa36:1	**PCaa38:3**	-0.61(-1.059,-0.16)	5.00E-05	3510.5	3.64
PCaa40:6	**PCae42:3**	22.993(5.487,40.5)	5.00E-05	3027	-2.48
PCaa38:4	**PCae40:4**	36.117(9.827,62.408)	4.00E-05	2999.7	-2.6
PCaa36:6	**PCae42:3**	0.125(0.035,0.216)	5.00E-05	2726.3	-2.8
PCaa38:3	**PCae40:6**	7.52(1.334,13.706)	0.00018	2614.6	-2.6
PCaa38:3	**PCaa38:4**	0.038(0.007,0.069)	2.00E-04	2231.1	-2.73
PCaa38:3	**PCae44:6**	151.9(24.75,279.1)	1.00E-04	2221	-2.09
PCaa32:1	**PCaa34:3**	-0.033(-0.06,-0.005)	0.00047	1989.4	2.22
PCaa38:3	**PCae42:2**	33.313(4.058,62.567)	0.00023	1869.6	-2.07
PCaa32:1	**PCaa38:3**	-0.017(-0.032,-0.001)	0.00024	1780.1	4.01
PCaa38:4	**PCae38:6**	25.419(4.684,46.153)	0.00015	1617	-2.5
PCaa36:4	**PCaa38:4**	-0.101(-0.211,0.009)	0.0017	1520	1.9
PCaa38:3	**PCae34:0**	133.72(18.055,249.38)	0.00013	1389	-2.11
PC.ae.C30.0	**PCae42:4**	0.163(0.042,0.284)	2.00E-05	1383.9	-2.47
PCaa38:3	**PCaa38:5**	0.312(0.042,0.583)	3.00E-04	1307.3	-2.4
PCaa38:3	**PCaa42:6**	28.374(2.981,53.767)	0.00034	1154	-1.99
PCaa38:4	**PCae38:0**	14.991(-0.118,30.1)	0.00108	1089.6	-2.81

PCaa38:3	**PCaa40:3**	22.593(1.77,43.415)	0.00036	1049.1	-2.03
PCaa38:3	**PCae44:5**	155.01(6.737,303.28)	0.00037	1020.3	-1.91
PCaa40:6	**PCaa42:5**	30.992(-1.641,63.625)	0.00148	1006.9	-1.92
PCaa36:2	**PCaa38:3**	-2.614(-5.056,-0.173)	0.00041	916.9	2.78
PCaa34:3	**PCae40:4**	1.97(0.35,3.589)	1.00E-04	872.4	-2.63
PCaa38:3	**PCae42:4**	82.87(22.864,142.8)	4.00E-05	857.9	-2.54
PCaa40:6	**PCae38:5**	3.002(0.184,5.819)	0.00079	814.7	-3.35
PCaa36:6	**PCaa38:3**	-0.003(-0.005,0)	0.00048	769.3	3.07
PCaa30:0	**PCaa38:3**	-0.005(-0.01,0)	0.00048	745.4	2.41
PCaa38:3	**PCae40:3**	21.223(-0.576,43.021)	0.00048	737.1	-1.59
PCaa40:6	**PCae40:5**	9.732(0.134,19.33)	0.00077	711.6	-2
PCaa36:5	**PCae40:4**	0.598(0.098,1.099)	0.00012	711.5	-2.47
PCaa38:3	**PCae36:2**	2.826(0.478,5.173)	0.00012	661.6	-2.28
PCaa38:4	**PCaa40:4**	10.54(-1.229,22.31)	0.00205	655.4	-1.84
PCaa36:2	**PCae34:2**	23.646(4.221,43.072)	0.00013	640.4	-2.47
PCaa38:0	**PCaa38:3**	-0.015(-0.029,-0.001)	0.00054	633.8	2.16
PCaa34:2	**PCaa38:3**	-2.814(-5.54,-0.089)	0.00055	606.3	2.65
PCaa32:3	**PCae40:2**	0.062(0.009,0.114)	2.00E-04	606.3	-2.38
PCaa38:6	**PCaa40:6**	-0.242(-0.55,0.066)	0.00483	531.5	1.56
PCaa38:4	**PCaa42:5**	118.85(-14.73,252.44)	0.00241	524.4	-1.83
PCaa38:3	**PCae44:4**	184.34(29.79,338.89)	8.00E-05	507.8	-1.98
PCaa38:4	**PCae38:5**	11.945(-0.28,24.169)	0.00115	500.7	-2.15
PCaa38:3	**PCae34:3**	25.206(4.209,46.203)	0.00018	470.7	-2.2
PCaa38:3	**PCae36:0**	83.644(9.256,158.031)	0.00013	397.8	-1.91
PCaa40:4	**PCaa40:6**	-0.009(-0.02,0.002)	0.00296	390.6	2.01
PCaa36:6	**PCae40:1**	0.013(-0.001,0.027)	0.00157	386.8	-2.82
PCaa34:3	**PCaa42:4**	9.597(-0.597,19.791)	0.00108	378.7	-1.75
PCaa38:3	**PCaa40:2**	29.938(-1.541,61.417)	0.00071	366	-1.6
PCaa38:3	**PCaa38:6**	0.093(0.003,0.182)	0.00083	363.4	-2.3
PCaa24:0	**PCae42:2**	0.175(-0.014,0.363)	0.00159	356.3	-1.56
PCaa38:3	**PCae40:2**	31.101(1.083,61.119)	0.00032	327.3	-1.75
PCaa34:3	**PCae34:2**	1.302(0.137,2.468)	2.00E-04	323.2	-2.3
PCaa24:0	**PCae42:4**	0.443(0.067,0.82)	1.00E-04	322.4	-1.99
PCaa34:3	**PCae38:4**	0.492(-0.075,1.06)	0.00215	292.3	-2.37
PCaa32:0	**PCaa34:3**	-0.148(-0.294,-0.002)	7.00E-04	288.9	2.76
PCaa34:3	**PCae36:3**	1.71(-0.307,3.728)	0.00271	288.8	-2.11
PCaa38:4	**PCae40:1**	12.202(-1.303,25.707)	0.002	277.9	-1.9
PCaa38:3	**PCaa42:2**	90.91(-0.911,182.73)	0.00055	247.3	-1.75
PCaa40:6	**PCae42:4**	68.42(10.632,126.2)	0.00013	235.5	-2.17
PCaa38:3	**PCaa40:5**	1.238(-0.016,2.493)	0.00118	229.3	-2.04
PCaa42:0	**PCae42:4**	0.171(0.029,0.313)	0.00013	228.1	-2.39
PCaa38:4	**PCae36:4**	13.704(0.401,27.008)	0.00085	222.4	-2.32
PCaa28:1	**PCaa40:2**	0.125(-0.001,0.252)	0.00105	217	-2.23
PCaa34:2	**PCae34:2**	24.335(2.273,46.397)	0.00028	198.2	-2.27
PCaa38:3	**PCae42:5**	32.126(0.546,63.706)	0.00054	185.3	-1.74
PCaa42:0	**PCaa42:2**	0.142(0,0.283)	7.00E-04	180.7	-2.53
PCaa34:3	**PCaa42:5**	6.931(-1.767,15.629)	0.00519	176.8	-2.21

PCaa40:6	**PCaa42:4**	42.522(-6.169,91.212)	0.00204	161.9	-1.59
PCaa34:3	**PCae36:2**	0.473(0.002,0.943)	0.00032	160.8	-1.86
PCaa34:1	**PCaa34:4**	-25.069(-53.105,2.966)	0.00166	159.2	2.61
PCaa32:3	**PCae40:4**	0.027(0.002,0.053)	0.00037	155.4	-1.82
PCaa38:5	**PCaa40:6**	-0.087(-0.215,0.04)	0.01064	150.5	1.49
PCaa38:3	**PCae42:1**	25.334(-3.336,54.004)	0.00163	149	-1.58
PCaa38:3	**PCae36:1**	9.776(-0.434,19.987)	0.00081	143.8	-1.71
PCaa34:3	**PCae36:4**	0.771(-0.062,1.604)	0.00122	139.4	-2.17
PCaa32:3	**PCae40:5**	0.028(-0.005,0.061)	0.00265	135.7	-1.53
PCaa24:0	**PCaa40:2**	0.152(-0.015,0.319)	0.00155	132	-1.56
PCae42:1	**PCae42:4**	0.46(0.061,0.859)	0.00018	131.7	-2.26
PCaa32:3	**PCae44:4**	0.418(0.041,0.795)	0.00024	125.6	-1.89
PCaa40:6	**PCae42:2**	22.051(-5.312,49.413)	0.00343	122.2	-1.48
PCaa34:4	**PCaa38:3**	-0.004(-0.009,0)	0.00189	120.6	2.02
PCaa34:3	**PCae38:6**	1.405(-0.092,2.901)	0.00115	112.4	-2.04
PCaa32:3	**PCae38:6**	0.019(-0.002,0.04)	0.00117	109.6	-1.6
PCaa24:0	**PCae32:2**	0.349(-0.079,0.777)	0.00479	108.8	-1.51
PCae38:4	**PCae40:4**	0.276(0.016,0.537)	5.00E-04	107.8	-2.02
PCaa32:3	**PCae42:4**	0.205(0.032,0.378)	0.00023	104.2	-2.39
PCaa34:3	**PCaa36:4**	0.006(-0.003,0.015)	0.01133	102.7	-2
PCae30:2	**PCae42:4**	0.113(0.008,0.217)	0.00024	100.3	-2.02
lysoPC20:3	**lysoPC20:4**	0.036(0,0.072)	0.00125	1376.3	-2.5
lysoPC18:1	**lysoPC20:3**	-1.616(-3.152,-0.079)	0.00032	396.7	3.5
lysoPC18:2	**lysoPC20:3**	-8.849(-17.873,0.174)	7.00E-04	396.5	2.49
SM(OH)22:1	**SM(OH)22:2**	0.72(0.306,1.134)	5.00E-05	17502.3	-3.47
SM(OH)22:1	**SM24:1**	0.154(0.056,0.253)	5.00E-05	7776	-3.08
SM(OH)22:1	**SM16:0**	0.032(0.011,0.053)	5.00E-05	4860	-2.86
SM(OH)14:1	**SM(OH)22:1**	-0.324(-0.537,-0.11)	2.00E-05	1407.7	3.75
SM16:1	**SM24:0**	-0.082(-0.169,0.005)	0.00145	947	2.36
SM(OH)16:1	**SM(OH)22:1**	-0.047(-0.079,-0.014)	5.00E-05	549.8	2.96
SM(OH)16:1	**SM18:0**	-0.025(-0.052,0.002)	0.00184	518.9	2.27
SM24:0	**SM24:1**	0.23(-0.046,0.505)	0.00274	402.4	-1.53
SM18:0	**SM24:1**	0.105(-0.011,0.222)	0.00236	370.6	-2.17
SM16:0	**SM24:0**	-0.449(-0.979,0.08)	0.00291	362	2.13
SM18:1	**SM24:1**	0.026(-0.007,0.058)	0.00433	216.7	-1.86
SM(OH)22:1	**SM16:1**	0.159(0.028,0.289)	0.00013	208.8	-2.53
SM(OH)14:1	**SM24:0**	-0.042(-0.098,0.013)	0.00548	149.1	1.54
PCae40:5	**SM24:0**	-0.028(-0.051,-0.004)	1.00E-04	9742.8	3.78
PCae36:3	**SM24:0**	-0.025(-0.047,-0.004)	0.00018	9459.4	2.43
PCae34:1	**SM24:0**	-0.037(-0.066,-0.007)	0.00023	7196.8	2.65
PCae40:2	**SM24:0**	-0.019(-0.033,-0.005)	2.00E-05	7170.1	4.22
PCae38:6	**SM24:0**	-0.073(-0.128,-0.019)	5.00E-05	5969.4	3.86
PCae40:2	**SM(OH)22:1**	-0.11(-0.184,-0.037)	5.00E-05	5790.6	4.97
PCae42:0	**SM24:0**	-0.011(-0.02,-0.002)	0.00015	5762.5	2.83
PCae34:2	**SM24:0**	-0.08(-0.136,-0.024)	2.00E-05	3852.7	3.17
PCae38:6	**SM(OH)22:1**	-0.413(-0.677,-0.149)	2.00E-05	2885.1	4.34
PCae38:3	**SM24:0**	-0.025(-0.049,-0.002)	0.00051	2822.8	2.59

PCae40:4	**SM24:0**	-0.063(-0.112,-0.014)	4.00E-05	2714.1	4.15
PCae38:0	**SM18:0**	-0.234(-0.469,0.001)	0.00054	2581.9	3.12
PCaa32:0	**SM24:0**	-0.199(-0.366,-0.031)	0.00013	2551.1	3.81
PCae44:4	**SM24:0**	-0.004(-0.007,-0.001)	2.00E-05	2458.1	3.11
PCae40:3	**SM24:0**	-0.015(-0.029,-0.001)	0.00054	2357.5	2.32
PCae44:4	**SM(OH)22:1**	-0.024(-0.04,-0.007)	2.00E-05	2200.8	3.77
PCae40:4	**SM(OH)22:1**	-0.343(-0.58,-0.105)	2.00E-05	1828.6	4.08
PCaa38:0	**SM24:0**	-0.043(-0.081,-0.005)	0.00028	1788.4	2.49
PCae38:5	**SM24:0**	-0.087(-0.168,-0.007)	5.00E-04	1495.6	2.94
PCae38:4	**SM24:0**	-0.112(-0.225,0.001)	0.00065	1419.9	2.77
PCae34:2	**SM(OH)22:1**	-0.457(-0.783,-0.131)	2.00E-05	1372.2	4.15
PCae40:5	**SM(OH)22:1**	-0.173(-0.295,-0.051)	2.00E-05	1316.9	3.91
PCae42:4	**SM(OH)22:1**	-0.065(-0.118,-0.013)	2.00E-05	1227.8	4.38
PCae34:3	**SM(OH)22:1**	-0.154(-0.263,-0.044)	4.00E-05	1033.5	4.13
PCaa36:2	**SM24:0**	-6.105(-12.585,0.375)	0.00137	1025.2	2.24
PCaa34:1	**SM24:0**	-2.978(-5.767,-0.188)	0.00045	889.4	2.5
PCaa42:4	**SM24:0**	-0.006(-0.013,0)	0.00057	867.8	2.86
PCae40:1	**SM18:0**	-0.408(-0.837,0.021)	0.00087	840	2.77
PCaa40:2	**SM24:0**	-0.018(-0.034,-0.001)	0.00038	811.1	2.69
PCae36:4	**SM24:0**	-0.103(-0.195,-0.01)	3.00E-04	805.6	2.73
PCaa42:5	**SM(OH)22:1**	-0.042(-0.076,-0.009)	4.00E-05	788.9	4.58
PCaa34:2	**SM24:0**	-6.68(-14.052,0.692)	0.00178	707.1	2.28
PCaa30:0	**SM24:0**	-0.014(-0.028,0)	0.00102	627.6	2.58
PCae38:4	**SM(OH)22:1**	-0.714(-1.246,-0.183)	5.00E-05	600.4	3.56
PCae36:3	**SM(OH)22:1**	-0.175(-0.307,-0.042)	5.00E-05	598.2	3.73
PCae38:4	**SM18:0**	-0.427(-0.866,0.012)	0.00129	579.5	2.58
PCae40:6	**SM18:0**	-0.16(-0.33,0.011)	0.00175	550.6	2.2
PCae36:2	**SM(OH)22:1**	-1.409(-2.439,-0.38)	5.00E-05	546.1	4.01
PCae40:1	**SM(OH)22:1**	-0.669(-1.251,-0.087)	5.00E-05	497.9	4.27
PCae38:6	**SM18:0**	-0.267(-0.514,-0.02)	0.00036	497.8	3.38
PCae38:0	**SM(OH)22:1**	-0.394(-0.718,-0.071)	5.00E-05	490.4	3.94
PCae42:2	**SM24:0**	-0.015(-0.032,0.003)	0.00142	416.9	3.32
PCaa42:5	**SM24:0**	-0.006(-0.014,0.001)	0.00276	397.3	2.72
PCae36:4	**SM(OH)22:1**	-0.611(-1.078,-0.144)	8.00E-05	392.5	3.41
PCaa34:1	**SM(OH)22:1**	-18.46(-33.15,-3.774)	8.00E-05	379.8	3.83
PCae42:3	**SM(OH)22:1**	-0.199(-0.373,-0.024)	8.00E-05	373.7	3.84
PCae40:6	**SM(OH)22:1**	-0.281(-0.501,-0.06)	8.00E-05	372	3.36
PCae36:2	**SM24:0**	-0.255(-0.465,-0.044)	0.00016	370.1	3
PCaa32:0	**SM(OH)22:1**	-1.246(-2.3,-0.192)	8.00E-05	356.5	4.39
PCaa42:5	**SM18:0**	-0.025(-0.054,0.004)	0.00244	353.9	2.75
PCaa32:3	**SM(OH)22:1**	-0.018(-0.032,-0.004)	8.00E-05	338.8	3.51
PCae36:1	**SM24:0**	-0.052(-0.101,-0.003)	0.00043	331	2.99
PCae42:4	**SM24:0**	-0.013(-0.025,-0.001)	1.00E-04	327.4	4.69
PCae34:3	**SM24:0**	-0.028(-0.052,-0.004)	0.00024	325.1	3.02
PCae38:5	**SM(OH)22:1**	-0.55(-0.967,-0.133)	8.00E-05	312.8	3.63
PCae40:6	**SM24:0**	-0.041(-0.094,0.011)	0.00324	308.9	2.76
PCaa42:2	**SM(OH)22:1**	-0.041(-0.076,-0.007)	8.00E-05	305.2	3.86

PCae38:5	**SM(OH)22:1**	-0.55(-0.967,-0.133)	8.00E-05	312.8	3.63
PCae40:5	**SM18:0**	-0.104(-0.217,0.008)	0.00159	272.8	2.43
PCaa38:3	**SM16:1**	1.563(-0.093,3.219)	0.00105	267.6	-1.72
PCaa36:1	**SM24:0**	-1.818(-3.365,-0.27)	0.00042	266.9	2.37
PCae42:3	**SM18:0**	-0.134(-0.266,-0.002)	0.00035	263.6	2.92
PCae42:5	**SM24:0**	-0.013(-0.025,-0.001)	0.00042	261.9	2.4
PCaa42:2	**SM24:0**	-0.007(-0.015,0)	0.00055	245.5	2.77
PCae38:0	**SM24:0**	-0.063(-0.141,0.016)	0.00322	244	3.27
PCaa40:4	**SM24:0**	-0.065(-0.148,0.017)	0.0039	240.2	1.94
PCae38:6	**SM18:1**	-0.483(-0.958,-0.007)	0.00064	238.8	2.95
PCae38:5	**SM18:0**	-0.337(-0.722,0.047)	0.00201	237.3	2.85
PCaa40:2	**SM(OH)22:1**	-0.107(-0.193,-0.021)	0.00012	236.3	3.61
PCaa40:5	**SM18:0**	-0.585(-1.299,0.129)	0.00342	216.7	2.41
PCae40:1	**SM24:0**	-0.113(-0.258,0.032)	0.00247	210.8	2.89
PCae36:5	**SM(OH)22:1**	-0.262(-0.474,-0.049)	0.00013	205.4	3.42
PCae36:1	**SM(OH)22:1**	-0.318(-0.584,-0.051)	0.00013	187.7	3.93
PCaa42:4	**SM(OH)22:1**	-0.041(-0.075,-0.006)	0.00013	172.1	3.11
PCae42:0	**SM(OH)22:1**	-0.074(-0.135,-0.014)	0.00015	169.4	3.01
PCae34:1	**SM(OH)22:1**	-0.254(-0.457,-0.05)	0.00015	164.4	3.23
PCaa38:5	**SM18:0**	-2.952(-6.69,0.785)	0.00423	162.1	2.2
PCae42:2	**SM(OH)22:1**	-0.088(-0.168,-0.007)	0.00015	161.7	3.55
PCaa36:3	**SM24:0**	-1.942(-4.454,0.57)	0.00523	159.7	1.51
PCae40:4	**SM18:0**	-0.223(-0.423,-0.022)	0.00039	144.9	3.04
PCae40:3	**SM(OH)22:1**	-0.103(-0.189,-0.017)	0.00015	144.5	3.05
PCae42:5	**SM(OH)22:1**	-0.083(-0.151,-0.016)	0.00015	143.2	2.96
PCaa38:6	**SM18:0**	-8.842(-20.221,2.538)	0.00466	140.2	2.19
PCaa38:0	**SM(OH)22:1**	-0.277(-0.513,-0.04)	0.00016	135	3.33
PCae44:6	**SM24:0**	-0.004(-0.007,0)	0.00087	135	2.22
PCaa36:4	**SM(OH)22:1**	-30.88(-57.30,-4.463)	0.00018	129.6	3.12
PCae38:6	**SM16:1**	-0.067(-0.136,0.002)	0.00108	121.7	2.92
PCae38:4	**SM18:1**	-0.703(-1.615,0.208)	0.00415	115.4	2.01
PCae42:2	**SM18:0**	-0.053(-0.12,0.014)	0.00373	109.2	2.12
PCae40:4	**SM18:1**	-0.413(-0.811,-0.016)	5.00E-04	109.1	2.69
PCae44:6	**SM(OH)22:1**	-0.022(-0.04,-0.003)	2.00E-04	105.4	2.99
PCae38:5	**SM18:1**	-0.556(-1.238,0.126)	0.0036	104.9	2.25
PCaa34:2	**SM(OH)22:1**	-52.076(-97.8,-6.351)	2.00E-04	104	3.19
lysoPC18:0	**PCaa38:3**	-0.736(-1.408,-0.063)	0.00051	687.2	2.3
lysoPC17:0	**PCaa38:3**	-0.012(-0.023,-0.001)	0.00072	436.3	2.23
lysoPC18:1	**PCaa38:3**	-0.329(-0.622,-0.036)	3.00E-04	423.6	2.98
lysoPC20:4	**PCaa38:3**	-0.343(-0.691,0.005)	0.00081	371	4.05
lysoPC16:0	**PCaa38:3**	-1.425(-2.854,0.004)	0.00098	292.5	2.18
lysoPC16:0	**PCae34:2**	16.855(2.016,31.694)	3.00E-04	183.8	-2.63
lysoPC28:0	**PCaa38:1**	0.146(-0.02,0.312)	0.00171	168.9	-1.53
lysoPC28:0	**PCae38:1**	0.273(0.029,0.517)	0.00013	146.4	-1.88
lysoPC26:0	**PCaa40:2**	0.29(-0.034,0.615)	0.00155	131.7	-1.68
lysoPC26:0	**PCaa38:1**	0.143(-0.025,0.311)	0.00218	122.4	-1.48
lysoPC28:0	**PCaa40:2**	0.297(-0.038,0.632)	0.00174	111.2	-1.71

lysoPC26:0	**PCaa42:2**	0.925(-0.078,1.928)	0.00105	107.1	-1.6
lysoPC17:0	**SM24:0**	-0.03(-0.064,0.004)	0.00277	265	1.74
C10	**SM16:1**	0.01(0.005,0.015)	5.00E-05	10613.5	-3.55
C4:1	**SM16:1**	0.004(0.002,0.006)	5.00E-05	3539.7	-4.13
C10	**SM(OH)14:1**	0.034(0.018,0.05)	5.00E-05	2392.8	-4.28
C10	**SM16:0**	0.002(0.001,0.003)	5.00E-05	1017.6	-3.43
C18	**SM(OH)14:1**	0.007(0.001,0.012)	0.00013	848.8	-2.26
C4:1	**SM24:1**	0.004(0.002,0.006)	1.56E-06	137.9	-3.25
C10	**lysoPC17:0**	0.056(0.027,0.084)	5.00E-05	6223	-3.35
C4:1	**lysoPC16:0**	0(0,0)	5.00E-05	2877.4	-3.53
C18	**lysoPC18:1**	0.001(0,0.001)	8.00E-05	1484.5	-2.53
C18	**lysoPC16:0**	0(0,0)	8.00E-05	1418.3	-3.05
C10	**lysoPC18:1**	0.004(0.001,0.006)	5.00E-05	471.4	-2.58
C5.OH(C3.DC.M)	**lysoPC18:2**	0(0,0)	0.00079	327.3	-1.61
C12:1	**lysoPC18:1**	0.001(0,0.001)	0.00026	152.3	-1.78
C4:1	**lysoPC24:0**	0.05(0.022,0.078)	1.56E-06	141.6	-2.85

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