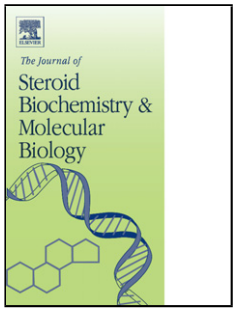

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Immediate reduction of serum citrulline but no change of steroid profile after initiation of metformin in individuals with type 2 diabetes

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Highlights

- **One dose of metformin already lowers serum citrulline in individuals with previously untreated type 2 diabetes.**
- **This effect is sustained for at least 4-6 weeks of metformin treatment.**
- **The steroid hormone profile is unchanged by metformin intake in subjects from the general population with type 2 diabetes - unlike previous findings in women with Polycystic Ovary Syndrome (PCOS).**

Abstract

Metformin is the most important first-line treatment for type 2 diabetes mellitus (T2DM) but its exact mode of action remains unknown. In this study, we used targeted metabolomics to gain new insights into the metabolic effects of metformin in humans with T2DM. We also examined changes in the serum steroid hormone profile. We quantified 167 serum metabolites and 19 steroid hormones using liquid chromatography-tandem mass spectrometry at three time points in individuals with previously untreated

T2DM: before the start of metformin therapy (time point A), after the first dose (B) and after short-term therapy for 4-6 weeks (C). For metabolite analysis, we split the study cohort into a discovery and a replication study of 88 and 45 subjects, respectively. The statistical analysis was done using linear mixed-effects models. Among the metabolites quantified, citrulline showed the most pronounced changes. Compared to its baseline serum concentration, citrulline was reduced by 17% after the first dose of metformin (p=1.34E-07) and by 24% after short-term therapy (p=2.84E-08) in the discovery study. These results were confirmed in the replication study. The only other metabolite significantly changed after correction for multiple testing was PC ae C36:4 between baseline and 4-6 weeks. The serum steroid hormone profile showed no significant changes after metformin intake. In summary, we observed an immediate and sustained reduction of serum citrulline by metformin in humans. This may be relevant for some of the wanted or unwanted effects of the drug.

Abbreviations.

AMPK	AMP-activated protein kinase
Arg	arginine
Asp	aspartate
Cit	citrulline
ESI-LC-MS/MS	electrospray ionization liquid chromatography-mass spectrometry
Orn	ornithine
OTC	ornithine transcarbamoylase
PC	phosphatidylcholine
PC aa	diacyl-phosphatidylcholine
PC ae	acyl-alkyl-phosphatidylcholine
PCOS	Polycystic Ovary Syndrome
SM	sphingomyeline
T2DM	type 2 diabetes

Keywords. drug treatment, targeted metabolomics, urea cycle, mode of action, phosphatidylcholine

1. Introduction

In 2013, the International Diabetes Federation estimated the worldwide diabetes prevalence at more than 8% and predicted an increase in the number of diabetes cases by 55% by 2035 [1]. Type 2 diabetes (T2DM) accounts for 85%–95% of all diabetes cases [1]. Therefore, improving treatment options for T2DM is of particular importance. Metformin has been in clinical use for more than 50 years and is recommended as the standard first-line oral antihyperglycemic agent. It is one of the few drugs available to pharmacologically treat insulin resistance [2,3]. It also reduces cardiometabolic risk factors [4]. However, clinical responses to metformin are variable. Metformin cannot be used in patients with renal insufficiency because of a risk of lactic acidosis, which represents a very rare but life-threatening complication [5,6]. Furthermore, about 20% of patients suffer from gastrointestinal side effects of metformin, which often lead to the discontinuation of treatment [7]. Therefore, there is a need to develop novel metformin-like pharmacologic agents. However, this process is hampered by the fact that the mode of action of metformin is not yet completely understood.

One molecular target for metformin is complex I of the mitochondrial respiratory chain [8]. Metformin impairs ATP production and activates AMP-activated protein kinase (AMPK). A decrease in energy charge enhances glycolysis and glucose uptake into cells and increases oxidation of fatty acids. This restores the energy balance, whereas energy demanding processes such as hepatic glucose production and lipid and cholesterol synthesis are inhibited [9-11]. However, the inhibition of complex I by metformin may also lead to an AMPK-independent reduction in cellular energy charge [9]. The molecular nature of these actions is unknown. Recently, metformin treatment of rats showed an increased cytosolic and decreased mitochondrial redox state due to inhibition of mitochondrial glycerophosphate dehydrogenase [12,13].

However, Baur et al. suggested that the alteration of the redox state during metformin treatment is rather caused by the production of NADH by cytosolic glycerol-3-phosphate dehydrogenase [14].

The investigation of drug-induced changes in the metabolome can help to better understand a drug's mode of action and the mechanisms of side effects [15,16]. To date, only three studies have investigated serum metabolite concentrations in humans with T2DM [15,17,18]. No study has yet looked at changes in metabolite concentrations after the very first dose of metformin and after short-term metformin monotherapy in the same individual. Therefore, the aim of this study was to determine changes in serum metabolites in previously untreated individuals with T2DM, after the first dose of metformin and after 4–6 weeks of treatment. We hypothesized that this approach would help to elucidate the drug's effects. Additionally, we investigated potential sex-specific effects of this drug on metabolism and examined its influence on the serum steroid hormone profile. As metformin is used successfully to lower serum androgen levels in women with Polycystic Ovary Syndrome (PCOS)[19], we hypothesized that it would also affect the steroid hormone profile of individuals with T2DM.

2. Materials and Methods

2.1. Study population

All study participants that were willing to participate were recruited through 51 medical practices (48 primary care practitioners, 3 diabetologists) in the state of Bavaria, Germany. Inclusion criteria were diagnosed T2DM, a HbA1c level between 6.0 and 12.0 percent, no antidiabetic drug therapy for at least three months, and a decision by the treating local physician to start metformin monotherapy. The study design was prospective and purely observational. All data and samples were collected at the offices of the local physicians, and all therapeutic decisions were made by the treating physicians. Of the 206 registered patients, 133 were finally included in the analyses. 52 were excluded because they did not meet all

inclusion criteria or due to incomplete blood samples or missing follow-up data. Further, eight study participants were excluded because no metformin was detected in the serum samples after the presumed first dose of metformin. 13 of the remaining patients (8.9%) discontinued metformin therapy in consultation with their physician due to persistent side effects (11 with gastrointestinal disorders and 2 with skin disorders). All participants in this study provided written informed consent. The study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the ethics committee of the Ludwig Maximilian University of Munich, Germany (no. 029-12). The characteristics of the study populations are shown in **table 1**.

2.2. Sample collection and preparation

Serum samples were collected from newly diagnosed T2DM patients after an overnight fast (discovery study $n = 88$, replication study $n = 45$; time point A). The first dose of metformin (500-1000 mg) was administered to each study participant between 6:00 and 9:00 PM within the following three days. The second fasting serum sample was collected the following morning, 10–16 h after the first dose of metformin (time point B). A third fasting serum sample was collected from a subgroup of 67 patients in the discovery study (30 males and 37 females) and 33 patients in the replication study (19 males and 14 females) 4–6 weeks after the initiation of metformin monotherapy (time point C). After individualized dose titration, the daily dose of metformin at time point C was between 1000 and 2000 mg. All blood samples were collected in 7.5 mL serum gel-barrier tubes with clotting activator (Sarstedt, Nuembrecht, Germany) and blood was coagulated for 30 min at room temperature. Samples from sites outside of Munich were centrifuged on-site, frozen at -20°C and transported to the central laboratory on dry ice within four weeks. Samples collected at sites in Munich were cooled down to 4°C, transported to the central laboratory within 2-6 h, centrifuged and separated in aliquots. In the central laboratory, serum samples were stored at -80°C until analysis.

2.3. Metabolite quantification

The targeted metabolomics approach was based on electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS/MS) and MS/MS measurements using the Absolute*IDQ*™ p180 assay (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay allowed simultaneous quantification of 188 metabolites in 10 µL of serum (For details see e-table 1 and [20]). Measurements were performed as described in the manufacturer's manual UM-P180. Analytical specifications for LOD and evaluated quantification ranges, further LOD for semiquantitative measurements, identities of quantitative and semiquantitative metabolites, specificity, potential interferences, linearity, precision and accuracy, reproducibility and stability were described in Biocrates manual AS-P180. The LLOQ and ULOQ were determined experimentally by Biocrates. The assay procedures of the Absolute*IDQ*™ p180 assay (including sample preparation and LC-MS/MS measurements) and the metabolite nomenclature have been described in detail previously [21,22]. Sample handling was performed using a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK). Mass spectrometric analyses were conducted on an API 4000 LC-MS/MS System (AB Sciex Deutschland GmbH, Darmstadt, Germany) equipped with 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Boeblingen, Germany) and HTC PAL auto samplers (CTC Analytics, Zwingen, Switzerland) and controlled by Analyst 1.5.1 software. For the calculation of metabolite concentrations, internal standards served as a reference. Concentrations of all metabolites were calculated in µmol/L. The analytical variance was determined by measuring metabolite concentrations of a reference sample of pooled human plasma with five replicates on each plate. In addition to the 188 absolute metabolite concentrations, 42 predefined sums and ratios were calculated using Met*IDQ*™ software (manufacturer's manual UM-RatioExplorer-1). Absolute metabolite concentrations and predefined sums and ratios are hereafter referred to as metabolite traits.

2.4. Steroid hormone quantification

Human serum samples (250 µL) were used for the analysis of steroids. The following 19 steroids were quantified using an extended version of the Absolute*IDQ*™ Stero17 assay and ESI-LC-MS/MS: aldosterone, androstenedione (androst-4-en-3,17-dione), androsterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate (DHEAS), dihydrotestosterone (DHT), estradiol, estrone, etiocholanolone, 17α-hydroxyprogesterone, progesterone, testosterone, pregnenolone, pregnanediol (last two steroids were assessed semiquantitative). Compound identification and quantification were based on scheduled multiple reaction monitoring measurements (sMRM). Sample preparation and LC-MS/MS measurements were performed as described in the manufacturer in manual UM-STERO17. We provide the description on how the method was implemented in the laboratory. Briefly, 400 µL of ultrapure water were pipetted into each well of a 2-mL-96-well deep well plate. Except into the blank well, 20 µL of the internal standard mix were added to each well. Thereafter, 250 µL of blank, calibration standards, quality control samples and plasma samples were pipetted into the distinct respective wells. The well content was mixed by aspiration using robot-driven pipets. In between, the SPE plate of the kit was conditioned successively with 1 mL of dichloromethane, followed by 1 mL acetonitrile, 1 mL methanol, and 1 mL ultrapure water. Except for sample loading, all SPE purification steps (conditioning, washing, drying, and eluting) were done by pressing solvents through the SPE plate using nitrogen and the positive pressure unit. The velocity was regulated by variation of the nitrogen pressure. After plate conditioning, the mixed samples were loaded onto the SPE plate. The samples dropped through very slowly by gravitation (1-2 drops per second). The SPE plate was washed with 500 µL water, dried for 1 h under nitrogen stream (58 psi). Steroids were subsequently eluted in two steps: 1) Two times with 500 µL dichloromethane into the same deep well plate (all steroids except DHEAS eluted), the eluate was dried each time for 20 min at 45 psi. 2) With 600 µL acetonitrile into another deep well plate. The first dichloromethane fraction was dissolved in 50 µL 25 % methanol and the plate was covered with a lid. To facilitate dissolving, the plate was treated for 1 min in an ultrasonic bath, and afterwards shaken for 5 min at 600 rpm. The second acetonitrile fraction was diluted with 400 µL

water and after placing a lid the plate was treated like the dichloromethane fraction. Both plates were centrifuged at 50 x g and placed into the cooled auto sampler (10 °C) for LC-MS/MS measurements. The LC-separation of both fractions was performed using 470 mL ultrapure water and the content of three ampules of the kit as mobile phase A and acetonitrile/methanol/ultrapure water v/v/v 85/10/5 as mobile phase B. Steroids were separated on the HPLC column for Absolute*IDQ*™ Stero17 assay combined with the precolumn SecurityGuard Cartridge C18 4 x 2 mm (for HPLC, Phenomenex Cat No. AJ0-4286). All solvents that have been used for sample preparation and measurement were of HPLC grade.

Analytical specifications for LOD (limit of detection), LLOQ and ULOQ (lower and upper limit of quantification), specificity, linearity, precision, accuracy, reproducibility, and stability were described in Biocrates manual AS-STERO17. Samples were handled using a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Waters Positive Pressure-96 Processor (Waters GmbH, Eschborn, Germany), beside standard laboratory equipment. Mass spectrometric analyses were done on a QTRAP 5500 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6. Based on internal standards, metabolite quantification and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex Deutschland GmbH, Darmstadt, Germany) as well as with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Kit.

2.5. Data preprocessing and quality control for metabolites

First, a plate correction was performed to correct for technical variation by multiplying each metabolite by a correction factor for each plate (geometric mean of the geometric means of the reference plasma samples across all plates divided by the geometric mean of the reference plasma samples of each plate). In total, 21 of the 188 metabolites were excluded from further analysis because they did not pass quality control

(> 50% of measurements at zero concentration or missing, or > 95% of all measurements below the limit of detection). The coefficient of variation (CV) within reference samples across all plates was > 25% for eight of the remaining 167 metabolites. These metabolites were not excluded, but labeled in the list of all investigated metabolite traits (electronic supplementary material e-table 1).

2.6. Statistical analysis

Changes in metabolite traits between time point A (before the first dose of metformin) and time point B (10–16 h after the first dose) and between time point A and time point C (after 4–6 weeks of metformin therapy) were determined using linear mixed-effects models (R package *nlme*, version 3.1-113 [23]], formula: $met_{ij} = time_{ij}*\beta_{time} + sex_i*\beta_{sex} + age_i*\beta_{age} + HbA1c_{ij}*\beta_{Hba1c} + b_i + \varepsilon ij$ where met_{ij} is the metabolite concentration measured for subject i at timepoint j and b_i a subject-specific intercept). Time point, sex, baseline HbA_{1c}, and age were modeled as fixed effects and sample identification as a random effect, and a two-sided t-test for zero time point effect was performed. A significance threshold of p = 1.21E-04 was used for the discovery study (Bonferroni correction for multiple comparisons). To investigate the presence of significant sex-specific effects, an interaction analysis was performed, in which sex*time point, sex*HbA_{1c}, and sex*age interactions were included as additional terms in the above described model. Significant sex*time point interaction was defined at p < 1.21E-04.

Metabolite traits that showed significant changes in either time point comparison were put forward to a replication step, using a significance threshold of p = 0.05 for the overall- and for the interaction analysis. To ensure that the observed significant associations were robust against violation of the distribution assumptions of the model, bias corrected and accelerated bootstrap confidence intervals were additionally calculated using 4,000 bootstrap samples [24]. For the analysis of serum steroids, the Wilcoxon signed-rank test with Benjamini-Hochberg correction for multiple testing was used. All statistical analyses were conducted with the R statistics platform, version 3.0.2 or with SAS, version 9.2.

3. Results

3.1. Impact of the first dose of metformin on metabolism

In the discovery study, we observed a significant change in citrulline (Cit) concentration between time point A (before the first dose of metformin) and time point B (10–16 h after the first dose), after correction for multiple testing. This metabolite trait was further investigated in the replication study and we achieved replication by showing significant changes between time points A and B (**table 2; figure 1**). No other metabolite was significantly changed between timepoints A and B. As Cit is in part formed in the urea cycle, additional information is given on metabolite traits that play a role within the urea cycle. Ornitine (Orn), arginine (Arg) and aspartate (Asp) were part of the analyzed metabolite panel but no significant changes in their metabolite levels were detected after the first dose of metformin. Furthermore, no significant changes of the systemic arginine bioavailability (ratio of arginine to sum of ornithine + citrulline) were shown between time points A and B.

Differences in the metabolomics response to the first metformin dose between male and female subjects were investigated in a sex-stratified analysis. Here, no significant differences were detected.

3.2. Impact of short-term metformin intake on metabolism

A third fasting serum sample was collected from a subgroup of participants (n = 66 in discovery and n = 34 in replication study) after 4–6 weeks of metformin monotherapy (time point C). We chose this time point because 4-6 weeks are usually required to titrate metformin to its final dose. In the discovery study, Cit and the phosphatidylcholine (PC) ae C36:4 showed a significant decrease between time point A and

time point C and changes in concentrations were confirmed in the replication study (**table 2**). The concentrations at time point C did not differ significantly from the concentrations at time point B (Cit: discovery: p = 0.40, replication: p = 0.71; PC ae C36:4: discovery: p = 2.69E-03, replication: p = 1.03E-03).

Besides Cit, further metabolite traits that play a role within the urea cycle were analyzed, namely Orn, Arg and Asp. Levels of these metabolite traits did not change significantly from timepoint A to C. Furthermore, no nominally significant changes between time points B and C were detected for the analyzed metabolite traits within the urea cycle and for the systemic arginine bioavailability.

No significant sex-specific effects were detected. All metformin-induced metabolic changes observed in the analyses were independent of sex, baseline HbA_{1c}, and patient age.

3.3. Impact of metformin intake on steroid hormone profile

We also examined the serum steroid hormone profile before, after the first dose and after 4-6 weeks of metformin intake. In the primary analysis, stratified for sex and, among female participants, for age (≤ 50 years vs. > 50 years), no significant changes of steroid hormones and calculated enzyme activities were observed (**e-table 3**). Similarly, we found no significant changes in an unstratified, secondary analysis (**e-table 4**).

4. Discussion

4.1. Impact of metformin on metabolism

The main finding of this study was a rapid and persisting reduction of the serum Cit level by metformin. Out of 167 investigated metabolites, only one further parameter was significantly changed by metformin after correction for multiple testing: PC ae C36:4 was reduced after 4 to 6 weeks of metformin.

The non-proteinogenic amino acid Cit is in part formed in the urea cycle by the condensation of Orn and carbamoylphosphate, catalyzed by Orn transcarbamoylase (OTC, EC 2.1.3.3) [25]. The main source of serum Cit however is the intestine. Approximately 80% of human serum Cit derives from glutamine converted in enterocytes [26]. Serum Cit therefore also serves as a marker of functional enterocyte mass in several clinical settings [27]. The main consumer of serum Cit is the kidney where the amino acid is utilized as an important precursor of Arg [28]. Arg in turn is used in nitric oxide production and this probably explains why oral Cit supplementation has a positive effect on vascular stiffness and blood pressure [29].

Based on these data the metformin-induced reduction of serum Cit could be the result of either a lower intestinal or hepatic Cit production or a higher renal Cit uptake. All three options could have clinical implications. A lower Cit production by the gut may be associated with the very common gastrointestinal side effects of metformin. Reduced hepatic Cit release could be related to changes in urea cycle activity, which itself is linked to gluconeogenesis [30]. Finally, metformin has been shown to increase endothelial nitric oxide production [31], which could result in higher utilization of the precursor Cit. Tracer studies will be required to clarify how the systemic flux of Cit is altered by metformin. Beyond that, it would be interesting to study metformin treatment together with Cit supplementation. Some effects of the drug may be more pronounced in that combination. Finally, the insulin sensitizer pioglitazone in combination with Metformin does not seem to alter its effect on serum Cit [32].

The significantly lower levels of the long-chain unsaturated acyl-alkyl phosphatidylcholine PC ae C36:4 after 4-6 weeks of metformin treatment, but not after the first dose of the drug, may be due to an improved metabolic status of the study participants and not to metformin per se. This would be in line with previous studies that described associations between altered PC levels and T2DM [33,34]. Furthermore, dietary intake of PC might be associated with incident T2DM risk [35]. Then et al. reported higher PC ae C36:4 fasting plasma levels in TCF7L2 risk allele carriers, which have a reduced first-phase insulin response and normal insulin sensitivity, compared to non-risk allele carriers [36].

4.2. Potential sex-based differences

Previous studies have identified sex-based differences in the activity and side effects of several drugs [37,38]. We investigated whether metabolic changes induced by metformin differed between male and female study participants, but detected no significant differences. Therefore, we suggest that metformin has comparable metabolic effects in both sexes. Sambol et al. [39] found no differences in metformin kinetics in male compared to female subjects.

4.3. Other studies of metformin-induced metabolic changes in humans

To our knowledge, this is the first study that used a targeted metabolomics approach to analyze the intra-individual immediate and short-term effects of metformin on metabolism in humans with T2DM. Our finding are in line with a recent study by Adam et al., which found reduced plasma Cit in individuals with T2DM on metformin therapy compared to those on dietary treatment, but did not include longitudinal comparisons in the same individual [18]. These authors also examined tissues of metformin-treated mice and found lower Cit in skeletal muscle and adipose tissue, but not in liver. Huo et al. [15] compared serum metabolites in T2DM patients after three months of metformin treatment with an untreated control cohort and found increased trimethylamine-N-oxide and 3-hydroxybutyrate, and decreased glucose, N-acetyl glycoprotein, lipoprotein, lactate, acetoacetate, and unsaturated lipids. In addition, they detected increased tryptophan and decreased lysoPCs (lysoPC a C16:0, lysoPC a C18:0 and lysoPC a C18:2) and phenylalanine in the metformin-treated group [15]. Some of these metabolites (tryptophan, lysoPC a C16:0, lysoPC a C18:0 and lysoPC a C18:2, and phenylalanine) were also measured in the present study but we found no significant differences in concentrations before and after treatment. The likely reasons for this discrepancy are the longer duration of metformin treatment and the between-subject analysis in the study by Huo et al., which differed from our within-subject approach. Another study of newly diagnosed T2DM patients compared serum samples before metformin treatment with samples taken 24 and 48 weeks after the initiation of treatment, and it found decreased serum glutamate after 24 and 48 weeks compared with

pretreatment concentrations [17]. Besides, in a study of Do et al. using a network-based approach, metformin showed a strong plasma link to Cit [40].

4.4. Metformin and serum steroid hormones

Unlike previous studies in women with PCOS [19], we did not observe metformin-induced changes in the serum steroid hormone profile in our study population. This result suggests that the hormone-modulating effects of the drug are specific for the hyperandrogenemic state of PCOS and not a general phenomenon. It also contradicts the notion that metabolic changes induced by metformin are secondary to alterations in steroidogenesis.

4.5. Strengths and limitations

Strenghts of this study are its cohort of individuals with drug-free T2DM and the available blood samples before and after the first dose of metformin. Limitations of the present study are its relatively small sample size and potential heterogeneity in data and biosample quality, due to the multicenter observational study design. Standard operating procedures were pretested and applied to limit these problems but residual heterogeneity nevertheless cannot be excluded. We employed targeted metabolomics because of its superior ability to quantify metabolites, in comparison to untargeted approaches. This approach may have resulted in undetected changes of metabolites not included in our panel. Additionally, as serum was the investigated matrix, it was not possible to distinguish from which tissue the detected metabolites derived. The here detected changes in metabolite ratios cannot directly predict enzyme activity. Thus, further analysis regarding enzyme activity need to confirm our results. Especially in multicenter studies, information regarding the stability of metabolites is of importance. Findings within the present study are unlikely to be the cause of inappropriate sample handling, as the reliability and pre-analytical stability of the investigated metabolites is high [20]. With respect to the serum steroid profile, our study has additional potential limitations. We only measured total, and not free testosterone and had no clinical information

available about menopausal status in female participants. We separated women aged 50 and younger from those older than 50 for our primary analysis but this represents only a rough estimate of menopausal status and therefore may have obscured effects present in only one of the two groups. We also had no information on menstrual cycle phase in premenopausal women. That precludes interpretation of the cycle-dependent hormones in the younger age group.

5. Conclusions

In summary, our objective was to use targeted metabolomics of serum samples to gain new insights into the metabolic effects of metformin in the treatment of T2DM. Our results indicate an link of metformin intake and Cit metabolism, which may contribute to some of drug's diverse effects. Further clinical and experimental studies are required to exactly clarify the underlying mechanisms.

Author contributions

M.B. researched and analyzed data, and wrote and edited the manuscript. S.W. discussed the data, and edited and reviewed the manuscript. C.P. performed the metabolomic experiments, and edited and reviewed the manuscript. U.F. designed the experiments, analyzed the data, and reviewed the manuscript. V.S. and M.W. contributed to the experiments and reviewed the manuscript. H.G. discussed the data, and edited and reviewed the manuscript. J.A. performed the metabolomic experiments, discussed the data, and edited and reviewed the manuscript. A.L. designed the experiments, researched and discussed the data, and edited and reviewed the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest that could be prejudicial to the impartiality of the research reported.

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figure 1. Citrulline levels before and after metformin intake.

Levels of citrulline at baseline (A), after the first dose of metformin (B), and after 4–6 weeks of metformin monotherapy (C) in the discovery and the replication studies.

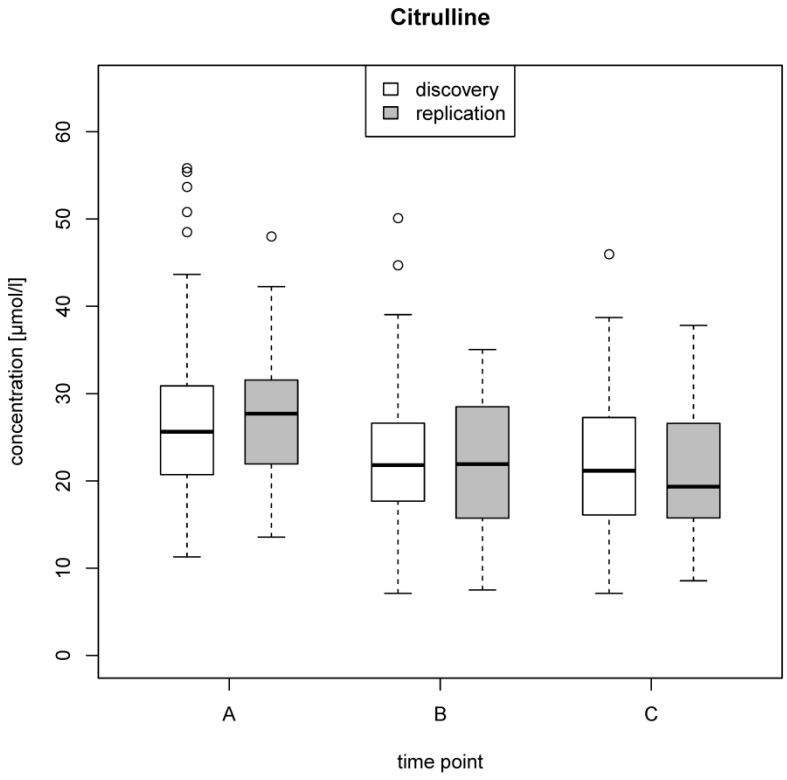

table 1. Clinical characteristics of the participants in the discovery and replication studies.

Characteristics	Discovery study	Replication study
Sample size (male/female)	88 (47/41)	45 (22/23)
Age[a] (years)	57 ± 12	63 ± 14
BMI[a] (kg/m^2)	32 ± 6	32 ± 6
HbA_{1c}[a] (%) (mmol/mol)	7.2 ± 1.2 (55.1 ± 12.7)	7.0 ± 0.9 (52.6 ± 9.5)

BMI; body mass index, HbA_{1c}; glycated hemoglobin. [a]Mean at baseline ± standard deviation

table 2. Changes in metabolite traits after the first dose and after short-term metformin therapy.

Beta estimates and *P*-values in the linear mixed effects regression model for significant changes in metabolite levels between time point A (baseline) and time point B (after the first dose of metformin) and between time point A and time point C (after 4–6 weeks of metformin therapy) in the discovery ($p < 1.21E-04$) and replication studies ($p < 0.05$). Cit, Citrulline.

	Metabolite trait	Cit		PC ae C36:4	
	time point comparison	A to B	A to C	A to B	A to C
Discovery study	beta estimate	-4.87	-5.70	-0.88	-2.20
	p-value	1.34E-07	2.84E-08	2.52E-02	1.05E-06
	baseline median concentration [µmol/l] ± change [%]	26.3 – 17.0	26.3 – 23.9	13.7-7.2	13.7-15.8
Replication study	beta estimate	-5.79	-6.27	-0.26	-1.99
	p-value	7.08E-06	9.27E-06	5.78E-01	1.93E-04
	baseline median concentration [µmol/l] ± change [%]	27.0 - 16.9	27.0 - 20.2	13.2-3.4	13.2-9.4