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First Time View on Human Metabolome Changes after a Single Intake of 3,4-Methylenedioxymethamphetamine in Healthy Placebo-Controlled Subjects

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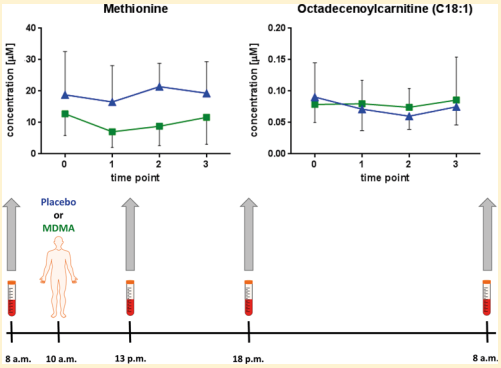
Supporting Information

ABSTRACT: 3,4-Methylenedioxymethamphetamine (MDMA; "ecstasy") is widely consumed recreationally. Little is known about its effects on the human metabolome. Mapping biochemical changes after drug exposure can complement traditional approaches by revealing potential biomarkers of organ toxicity or discovering new metabolomic features in a time- and dose-dependent manner. We aimed to analyze for the first time plasma samples from a randomized, double-blind, placebo-controlled crossover study in healthy adults to explore changes in endogenous plasma metabolites following a single intake of MDMA. Plasma samples from 15 subjects taken at four different time points were analyzed with the commercially available AbsoluteIDQ kit (Biocrates). Time series analysis revealed a total of nine metabolites, which showed a significant concentration change after MDMA administration compared with placebo. Paired *t* tests of the single time points showed statistically significant concentration changes mainly of glycerophospholipids and the metabolic ratio of methionine-sulfoxide over methionine. Changes of this metabolic ratio may be indicative for changes in systemic oxidative stress levels, and the increased amount of glycerophospholipids could be interpreted as an upregulation of energy production. Baseline samples within the experimental study design were crucial for evaluation of metabolomics data as interday individuality within subjects was high otherwise resulting in overestimations of the findings.



KEYWORDS: *3,4-methylenedioxymethamphetamine (MDMA), ecstasy, targeted metabolomics, biocrates, methionine, placebo-controlled*

INTRODUCTION

The recreational psychoactive drug 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy") is an indirect monoaminergic agonist that releases serotonin (5-hydroxytryptamine; 5-HT) and norepinephrine (NE) and to a lower extent dopamine (DA), presynaptically by interacting with the corresponding membrane transporters.[1−3] MDMA produces feelings of positive mood, closeness, and prosociality[4,5] but also sympathomimetic toxicity (e.g., tachycardia, hypertension, and hyperthermia[6,7]), an increase in metabolic rate,[8] and an acute endocrine stress response with elevated plasma concentrations of cortisol, oxytocin, and arginine vasopressin.[5,9−12] However, little is known about additional effects of MDMA on the human metabolome.[13]

Metabolomics research focuses on high-throughput identification of small molecular weight molecules. This technology allows measurements of a multitude of metabolites in one single sample. Mapping the biochemical changes after drugs of abuse exposure may complement traditional approaches by revealing potential biomarkers of organ toxicity, discovering new metabolites in time- and dose-dependent manner and different pharmacodynamic targets, as well as by giving insights about the pathways implicated in the mechanism of action, adverse effects, and variability of the drug response.[14] From the analytical point of view, detection of new biomarkers attributable to drugs of abuse consumption might serve as alternative hint of their consumption particularly useful in case of short detection windows or new psychoactive substances with similar mechanisms of action. Information on influence of acute or chronic drugs of abuse consumption on the metabolome is limited. Targeted cardiac metabolic profiling in rat heart tissue after different MDMA doses revealed increased carnitine and decreased choline levels.[15] First attempts of metabolic profiling studies after MDMA consumption in humans were done by Nielsen et al.[16] They investigated retrospective forensic data from whole blood samples of humans exposed to MDMA with an untargeted

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metabolomics screening analysis.[16] The downside of this study was that no information about drug use history, time between MDMA administration and blood sampling, or food intake of the subjects was available.

One of the largest challenges of metabolomics is to overcome interindividual and intraindividual metabolite variations. Metabolites can not only be directly produced and influenced by the host organism, but also from xenobiotics, diet and lifestyle habits, genetic factors (gender, genotype, age), and other exogenous sources.[17] These influences result in significantly different metabolic responses, making it difficult to find metabolites that are correlated with a particular condition. Therefore, it is important to establish an appropriate experimental study design with controlled conditions to identify metabolites that exclusively correlate with the intake of a specific biological stimulus. We analyzed for the first time unique plasma samples from a randomized, double-blind, placebo-controlled crossover study using administration of MDMA at a defined dose of 125 mg and placebo in young healthy adults. Each participant served as his or her own control, which limited the effects of intraindividual variability. The goal of this study was to explore changes in plasma metabolites in response to a single intake of MDMA and compare these results to metabolic changes in the same subjects after placebo ingestion. For the analysis of the plasma samples, the commercially available targeted metabolomics approach AbsoluteIDQ from Biocrates was used.

EXPERIMENTAL SECTION

Clinical Study and Sample Collection

Stored plasma samples (−80 °C, maximal time 2.5 years) from a double-blind, placebo-controlled crossover study were used.[18] The samples were aliquoted upon arrival at our institute and did not undergo more than two freeze−thaw cycles. In brief, 16 healthy subjects (eight male and eight female subjects, age 20−27) were recruited with known drug use history where MDMA pills were consumed once in their lifetime by six participants. Further prevalence of drug use and details of the study with participant inclusion criteria has been described previously.[18,19] The clinical crossover study included four test sessions, but for this metabolomics study, samples from only two sessions, the placebo and the MDMA alone sessions, were used. The duration of the wash-out periods between the sessions was at least 10 days. On the session day, MDMA (125 mg p.o., corresponding to 1.8 ± 0.2 mg/kg body weight) or placebo was administered at 10 a.m. Plasma samples were collected 2 h before at 8 a.m. (= time point 0) and 3 h (= time point 1), 8 h (= time point 2), and 24 h (= time point 3) after administration of MDMA or placebo. Plasma was collected at additional time points for pharmacokinetic analyses not used in the present study and are reported elsewhere.[18,19] During the session days, participants were under surveillance, and they were served a standardized meal at 12:30 p.m. The clinical study was conducted at the University Hospital of Basel in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice. It was approved by the Ethics Committee of the Canton of Basel, Switzerland, and the Swiss Agency for Therapeutic Products (Swissmedic). The study was registered at ClinicalTrials.gov (NCT01771874). All subjects provided written informed consent and were paid for their participation.

Sample Preparation and Analysis

A targeted metabolomics approach of combined liquid chromatography (LC) and direct flow injection analysis (FIA) using the AbsoluteIDQ p180 kit (Biocrates, Life Sciences AG, Innsbruck, Austria) was used. The kit has been validated by Biocrates for human blood plasma following FDA criteria and the analytical specifications accuracy, precision, linearity, stability, and reproducibility of the quantification of the metabolites are described in the analytical specifications.[20] It allows for a simultaneous quantification of up to 188 metabolites from the six compound classes amino acids (AA), biogenic amines, acylcarnitines, carbohydrates, and phospho- and sphingolipids. The nomenclature of the lipid metabolites is as follows: acylcarnitines (C*x*:*y*), hydroxyacylcarnitines [C(OH)*x*:*y*] dicarboxylacylcarnitines (C*x*:*y*-DC), sphingomyelina (SM*x*:*y*), sphingomyelin derivatives [SM(OH)*x*:*y*], lyso- and phosphatidylcholines (PC). The side chain composition of the lipids is indicated with 'C*x*:*y*', whereby '*x*' describes the number of carbons in the side chain and '*y*' the number of double bonds. Lyso- and phosphatidylcholines are further differentiated to the presence of ester ('a') and ether ('e') bonds in the glycerol moiety. A single letter 'a' (= acyl) indicates the presence of a single fatty acid residue in the molecule. Two letters "aa" (= diacyl) and "ae" (= acyl-alkyl) indicate two glycerol positions bound to a fatty acid residue.[21] A list of all analyzed metabolites can be found elsewhere.[22]

Briefly, plasma samples were randomized, and 10 μL of every sample was transferred onto a 96-well kit plate containing the stable isotope labeled internal standards (IS). The samples were dried under a nitrogen stream at room temperature and derivatized with 5% phenylisothiocyanante reagent. After a second drying step, samples were extracted with 5 mM ammonium acetate in methanol and centrifuged through the filter plate (2 min, 500*g*). Samples were diluted with the solvent mixture used for ultrahigh pressure liquid chromatography−tandem mass spectrometry (UHPLC−MS/MS).

A Thermo Fischer Ultimate 3000 UHPLC system (Thermo Fisher, San Jose, California, USA) coupled to a Sciex 5500 QTrap linear ion trap quadrupole MS (Sciex, Darmstadt, Germany) was used. The MS was controlled by Analyst 1.6.2 software. An LC gradient elution was performed using a Waters UPLC BEH C18 column (2.1 × 75 mm^2, 1.7 μm) with water containing 0.2% (v/v) formic acid (eluent A) and acetonitrile containing 0.2% (v/v) formic acid (eluent B).

Metabolites from the AA and the biogenic amine groups were quantified by multiple reaction monitoring (MRM) injecting 10 μL of the sample in the UHPLC−MS/MS system. Flow injection analysis (FIA-MS/MS) was used to quantify acylcarnitines, lipids, and hexoses. The sample was injected twice for negative and for positive electrospray ionization mode. The MetIDQ software package (version 5.5.4), which is an integral part of the kit, was used for quantification of the metabolite concentrations, validation, and data evaluation. Eighty plasma samples were measured with one kit, and in total three kits were used to measure all samples of 15 out of 16 participants from the study. The three kits used for measurements were all from the same batch. To minimize kit-plate effects, all samples from the same participant were measured on the same plate. Three replicates of a quality control (QC medium) were included on every kit plate and evaluated for the present study for intra- and interplate precision.

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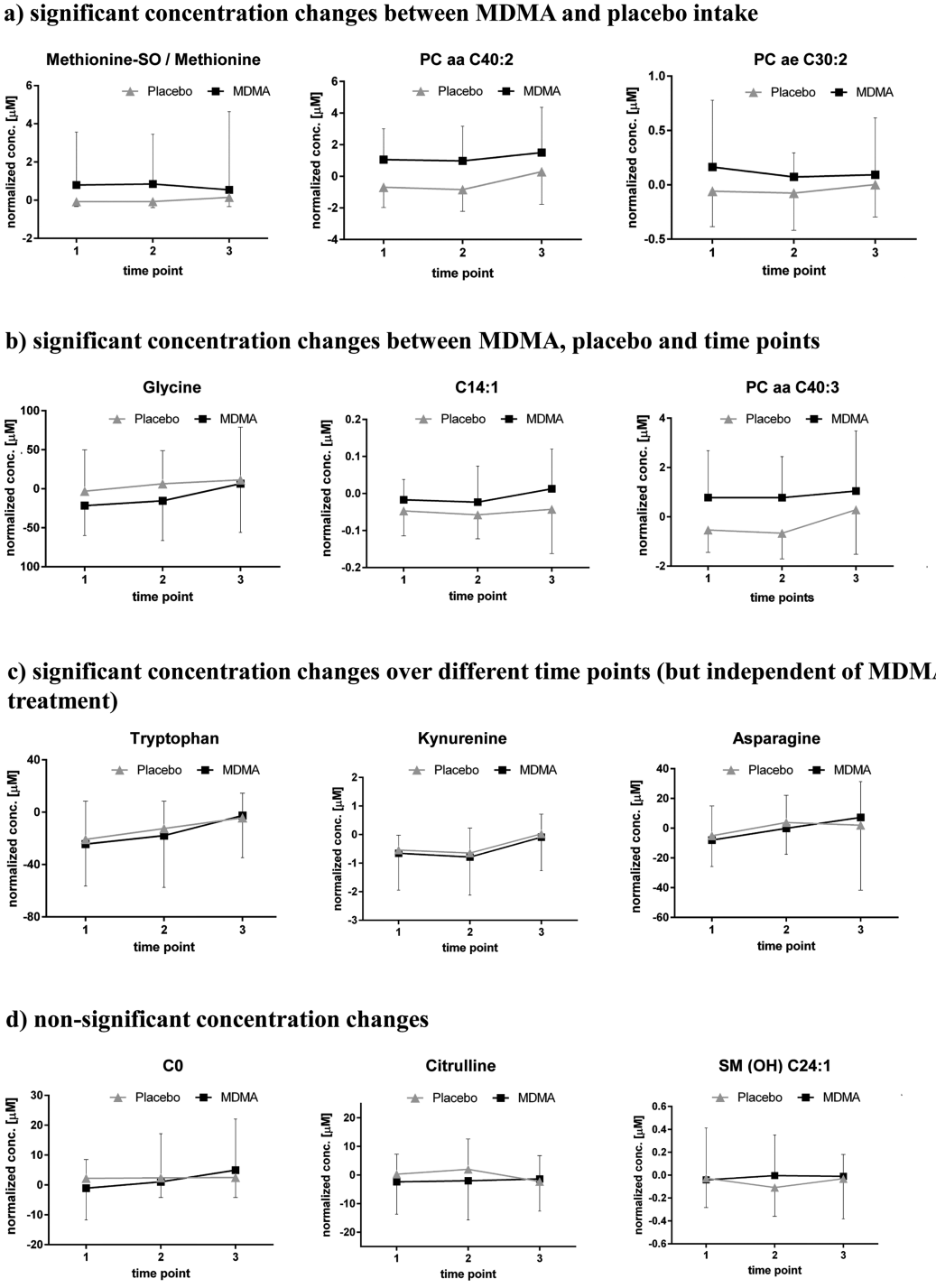

Figure 1. Time series of selected metabolite concentrations comparison between the sessions at the different baseline-corrected time points: (a) metabolites with significant concentration changes between placebo and MDMA session, (b) metabolites with significant concentration changes between placebo and MDMA session and time points differences, (c) metabolites showing statistically significant changes between the time points but independent of MDMA treatment; (d) metabolites with no significant concentration changes between placebo and MDMA session. Placebo condition is indicated by gray triangles, MDMA conditions by filled squares, respectively. Data points are given as mean and standard deviation of 15 participants. Statistical tests were performed by two-way repeated measure ANOVA with $p < 0.05$ indicating significant differences including false discovery rate for multiple test corrections.

Statistical Methods

Data Preprocessing. Measured concentrations were normalized to median with the QC medium samples measured on every kit plate and log-transformed to obtain normal distributed metabolites. After normalization, the data were checked for normality with $q-q$ plots. Most of the metabolites showed normal distribution, and parametric tests were used for further analyses. One plasma sample from time point 2 (8 h after placebo intake) of the placebo session was missing, and the corresponding sample from the same participant 6 h after placebo intake was measured and included in the calculations. Metabolites appearing in less than 80% of the samples were

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Table 1. Summary of Significantly Altered Metabolites between Placebo and MDMA Session

	uncorrected values		baseline-corrected values			
	tp 0		tp 1 (tp 1 − tp 0)		tp 2 (tp 2 − tp 0)	
metabolite	significance[a]	paired fold change	significance[a]	paired fold change	significance[a]	paired fold change
Asn	n.s.		*	1	n.s.	
Gly	n.s		n.s.		*	1
Met	***	1.2	n.s.		n.s.	
Met-SO	*	1.2	n.s.		n.s.	
Met-SO/Met	***	1.6	***	>5	**	3.1
Tyr/Phe	n.s.		n.s.		*	1
C16	n.s.		n.s.		*	1
C18:1	n.s.		n.s.		*	1
Diacyl-phosphatidylcholines						
PC aa C26:0	n.s.		n.s.		*	1
PC aa C36:0	***	1.4	n.s.		*	1
PC aa C38:0	n.s.	1.2	n.s.		*	1
PC aa C38:1	*	1.3	n.s.		*	1
PC aa C38:6	*	1.1	n.s.		n.s.	
PC aa C40:1	**	1.1	**	>5	**	>5
PC aa C40:2	*	1.1	***	2.1	*	>5
PC aa C40:3	n.s.		**	1	**	>5
PC aa C40:4	n.s.		*	1	**	1.3
PC aa C42:0	n.s.		**	1	**	1
PC aa C42:1	*	1.1	***	>5	*	1
PC aa C42:2	**	1.1	***	>5	**	3.6
PC aa C42:4	n.s.		*	1	**	1
PC aa C42:5	n.s.		*	1	*	1.2
PC aa C42:6	n.s.		n.s.		*	1
Acyl-alkyl-phosphatidylcholines						
PC ae C30:2	**	1.1	n.s.	3.1	n.s.	
PC ae C32:2	n.s.		n.s.		*	1
PC ae C36:1	*	1.1	**	1	n.s.	
PC ae C38:1	**	1.1	**	1	n.s.	
PC ae C38:2	*	1.2	***	1	n.s.	
PC ae C38:5	*		n.s.		n.s.	
PC ae C38:6	*	1.3	n.s.		n.s.	
PC ae C40:1	*	1.1	*	1	*	1
PC ae C40:2	n.s.		***	1.8	*	1
PC ae C40:3	n.s.		n.s.		*	1
PC ae C40:6	*	1.1	n.s.		n.s.	
PC ae C42:1	**	1.1	**	1	*	>5
PC ae C42:2	n.s.		**	1.6	***	>5
PC ae C42:3	n.s.		**	4.5	*	1.8
PC ae C42:4	n.s.		*	1	n.s.	
PC ae C42:6	n.s.		n.s.		n.s.	
PC ae C44:3	n.s.		***	>5	*	1
PC ae C44:4	n.s.		n.s.		*	1
PC ae C44:6	n.s.		*	1	n.s.	
Lyso-Phosphatidylcholine acyl						
lysoPC a C26:1	n.s.		n.s.		*	1
lysoPC a C28:1	n.s.		n.s.		*	>2

[a]Statistical significance with FDR < 0.05 and * $p \leq 0.01$ and $p \geq 0.001$, ** $p < 0.001$ and $p \geq 0.0001$, *** $p < 0.0001$; n.s., not significant.

removed from further statistics. Remaining missing data and concentrations under the detection limit were replaced by half of the minimum positive value in the original data. Additionally, 19 metabolic ratios were calculated and included in the statistical analysis (Supporting Information).

Statistical Calculations. All statistical tests were performed by the open source statistical software R (3.2.4) and the web-tool metaboanalyst 3.0.[23]

Metabolite concentrations at time point 0 were set as baseline concentrations to minimize interday variabilities. Therefore, the baseline-corrected data differences were calculated for all analytes between time point 1 (3 h), 2 (8 h), and 3 (24 h) and time point 0 in each session, respectively. As an example:

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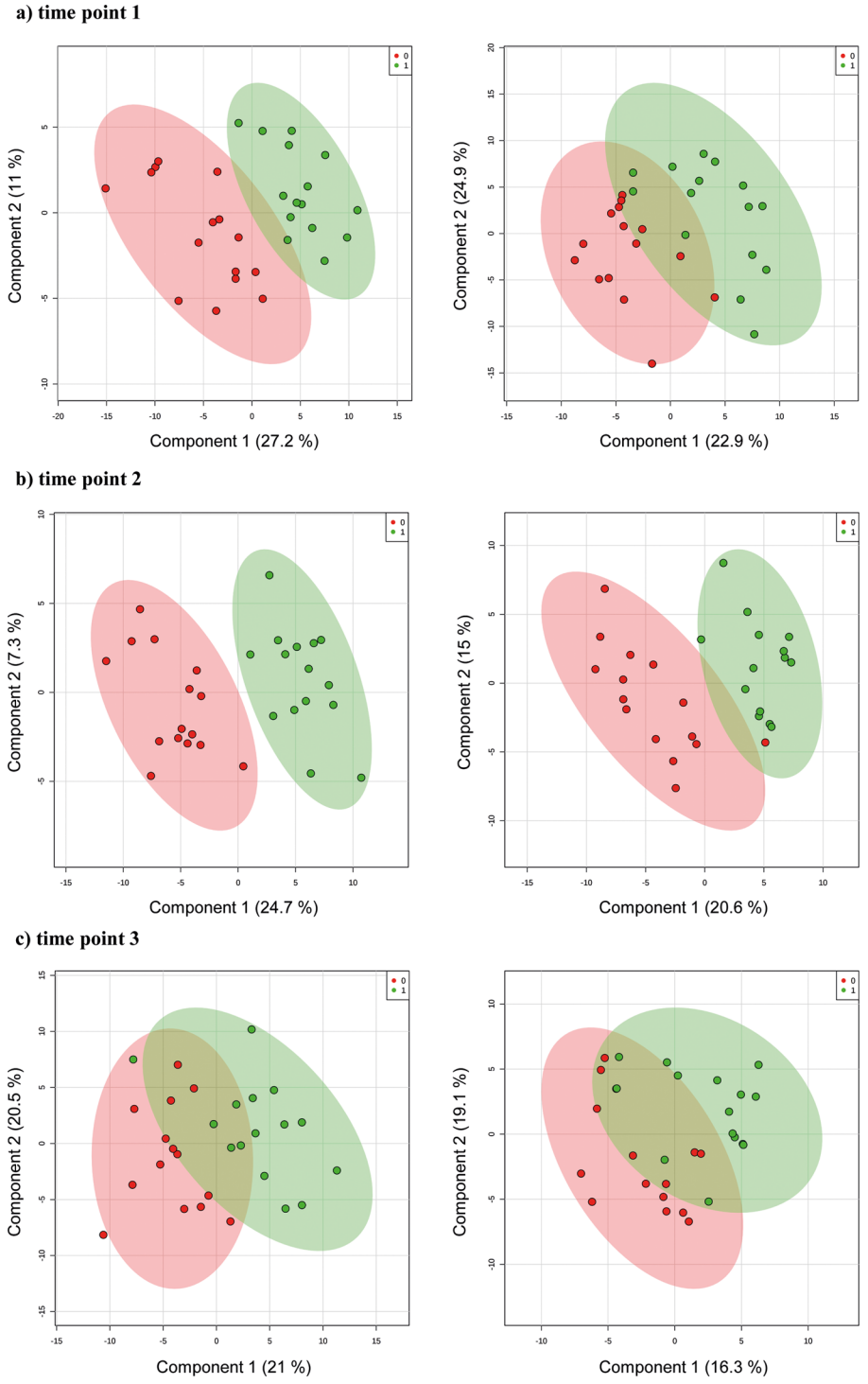

Figure 2. PLS-DA scores plot at the different time points (a) 3 h, (b) 8 h, (c) 24 h after MDMA administration. MDMA (green) or placebo (red) intake for 15 participants and resulting 95% confidence region. Left column represents noncorrected data set, right column represents baseline-corrected data sets to tp = 0 (2 h prior to MDMA or placebo intake).

- $x_{iM\text{-}t0}$ and $x_{iM\text{-}t3}$ be samples from participant i at time point 0 (baseline) and 3 (24 h) acquired during the MDMA (M) session.
- $x_{iP\text{-}t0}$ and $x_{iP\text{-}t3}$ are the corresponding samples during the placebo (P) session.

difference: $d_{xiM-t3-t0} = x_{iM-t3} - x_{iM-t0}$

difference: $d_{xiP-t3-t0} = x_{iP-t3} - x_{iP-t0}$

Paired fold change analysis was calculated from non-transformed plasma concentrations. The fold change was calculated as the concentration ratio between the MDMA and the placebo session. A threshold of 75% was applied meaning that a minimum of 11 paired samples needed a consistent change above the given fold change threshold. Paired t tests of noncorrected as well as baseline-corrected data were conducted to identify statistically significant ($p < 0.01$) within-person differences. False discovery rate (FDR) methods were

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used to correct the data for multiple testing.[24] For time series analysis, baseline-corrected metabolites were further tested with two-way repeated measure analysis of variance (ANOVA) (within subject), and FDR correction for multiple testing was applied.

Multivariate Analysis. After the evaluation of different scaling methods, autoscaling was applied prior to principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). PCA was used as a visualization tool for the data and to detect possible outliers and batch effects. Multilevel PLS-DA was carried out for class discrimination to investigate whether the MDMA intake led to significant metabolic changes 3 h, 8 h, or 24 h after consumption. PLS-DA was performed in combination with cross model validation (CMV) and permutation testing to validate if prediction error of the PLS-DA model was not obtained by chance. Prediction errors from 1000 randomly permutated data sets were collected and represented as the H_0-distribution of no-effect. MDMA intake was considered statistically significant if the p-value of the permutation test was $p \leq 0.05$. Variable importance parameters (VIPs) from PLS-DA summarized the importance of each metabolite to contribute to the separation of the two groups with VIP ≤ 1 being unimportant and VIP ≥ 1.5 being largely important.

RESULTS

We profiled 188 metabolites in blood plasma of 15 study participants from a controlled MDMA administration study to humans. After preprocessing, 141 metabolites (6 acylcarnitines, 21 AA, 13 biogenic amines, 88 glycero-phospholipids, 12 sphingolipids, 1 sugar) passed the quality control and were subject to further statistical analysis.

Analytical between batch coefficients variation (CV) of six metabolites (serine, 5-HT, spermine, symmetric dimethylarginine (SDMA), PC aa C34:1, and SM C18:0) was greater than 20%, and these metabolites were excluded from the statistical calculations. Interday and intraday repeatabilities of QC medium were calculated and were less than 20% for all metabolites except for interday repeatability of SDMA (25.7%) and PC aa C34:1 (24.9%).

Metabolite Pool Size Changes

All baseline-corrected metabolites were specifically tested with two-way repeated measure ANOVA (within subject), and FDR for multiple testing corrections was applied. ANOVA revealed nine metabolites, which showed a significant ($p < 0.05$) concentration change between the two sessions (Figure 1a), 23 metabolites showed a significant concentration change between the sessions and the time points (Figure 1b) and 88 metabolites showed a significant concentration-change between the time points independent of MDMA administration (Figure 1c), respectively. Forty metabolites showed no changes between the two sessions and the time points (Figure 1d). The metabolites that showed changes between the sessions were PC aa (C26:0, C38:1, C40:1), PC ae (C30:2, C42:2), C18:1, lysoPC a (C26:1, C28:1), and the ratio methionine sulfoxide (Met-SO) over methionine (Met) (Met-SO/Met). Many AA showed only significant concentration changes between the different time points, namely Met, phenylalanine (Phe), threonine (Thr), histidine (His), tyrosine (Tyr), Met-SO, asparagine (Asn), arginine (Arg), leucine (Leu), isoleucine (Ile), lysine (Lys), aspartic acid (Asp), glutaminc acid (Glu), tryptophan (Trp), valine (Val), glutamine (Gln), alanine (Ala), Taurine, Creatinine, Kynurenine (Figure 1).

Effect of MDMA Intake at Different Time Points

Time points of both sessions were compared using PCA, PLS-DA, and paired t test. To evaluate if the baseline time point (tp 0) has a great effect on the statistical outcome, the calculations were done twice: on the noncorrected time points as well as on interday variability corrected time points where the respective deviations of tp 1, tp 2 or tp 3 minus tp 0 were used. Metabolites with $p < 0.01$ and FDR < 0.05 were considered to be statistically significant. A summary of all statistically significant metabolites is given in Table 1.

Multivariate analysis using PCA showed no clear clustering between the MDMA and placebo intake at any of the measured time points. The clustering according to the individual participants showed that interindividual variability was high.

Concentration Differences 2 h (= Time Point 0) before MDMA or Placebo Intake. The concentrations of the 19 metabolites PC aa (C36:0, C38:1, C38:6, C40:1, C40:2, C42:1, C42:2), PC ae (C30:2, C36:1, C38:1, C38:2, C38:5, C38:6, C40:1, C40:6, C42:1), Met, Met-SO, and the ratio Met-SO/Met were statistically different between the two sessions before the intake of MDMA or placebo, respectively, indicating presence of high interday variabilities (Table 1). PLS-DA showed no clear separation of the two groups with non-significant cross validation and permutation-testing.

Concentration Changes 3 h (= Time Point 1) after MDMA or Placebo Intake. Paired t test of uncorrected metabolites showed that concentration of 64 metabolites significantly changed between the two sessions. Permutation testing for PLS-DA showed no significant result ($p = 0.819$) indicating an overfitting of the model (Figure 2).

Paired t test with interday corrected values showed significant concentration differences in 23 metabolites between the two sessions, mainly glycerophospholipids PC aa (C40:1, C40:2, C40:3, C40:4, C42:0, C42:1, C42:2, C42:4, C42:5), PC ae (C36:1, C38:1, C38:2, C40:1, C40:2, C42:1, C42:2, C42:3, C42:4, C42:6, C44:3, C44:6), Asn, and the metabolic ratio Met-SO/Met (Table 1). PLS-DA showed no significant separation of the two sessions (Figure 2).

Concentration Changes 8 h (= Time Point 2) after MDMA or Placebo Intake. With the noncorrected data set, a total of 64 metabolites showed significant differences with paired t testing between the two sessions. PLS-DA was able to separate the two groups with $R^2 = 0.88$ and $Q^2 = 0.75$ showing a good model (Figure 2). The p-value ($p = 0.007$) obtained from this permutation test with 1000 permutations demonstrates the validity of the model. A total of 29 metabolites had a VIP value >1.5, mainly glycerophospholipids: 11 PC aa (C24:0, C36:0, C38:0, C38:1, C40:1, C40:2, C40:3, C42:0, C42:1, C42:2, C42:6), 11 PC ae (C30:2, C36:1, C38:1, C38:2, C40:1, C40:2, C42:1, C42:2, C42:3, C44:3), five lysoPC a (C24:0, C26:0, C26:1, C28:0, C28:1) as well as Met, Met-SO and the metabolic ratio Met-SO/Met.

Paired t test of the corrected data set revealed 30 metabolites to be significantly different between the sessions, namely PC aa (C26:0, C36:0, C38:0, C38:1, C40:1, C40:2, C40:3, C40:4, C42:0, C42:1, C42:2, C42:4, C42:5, C42:6), PC ae (C32:2, C40:1, C40:2, C40:3, C42:1, C42:2, C42:3, C44:3, C44:4), lysoPC a (C26:1, C28:1), C16, C18:1, Gly, Met-SO/Met, Tyr/Phe (Table 1). Although cross-validation testing ($R^2 = 0.89$, $Q^2 = 0.59$) showed a relatively good data fit of the PLS-DA model,

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permutation testing revealed no significant separation between both sessions (P = 0.893) indicating an overfit of the model (Figure 2).

Concentration Changes 24 h (= Time Point 3) after MDMA or Placebo Intake. Paired t test of the uncorrected data showed a significant concentration change in 42 metabolites. PCA detected one sample as outlier, which was excluded from PLS-DA. PLS-DA showed no complete separation between the MDMA and the placebo session. However, the cross-validation of the model showed a R^2 = 0.54 and Q^2 = 0.29, and permutation tests showed a significant result of p = 0.005. A total of 30 metabolites had a VIP value >1.5, mainly glycerophospholipids, 11 diacyl-phosphatidylcholines: PC aa (C24:0, C36:0, C40:1, C40:2, C40:3, C42:0, C42:1, C42:2, C42:6), 11 acyl-alkyl-phosphatidylcholines: PC ae (C30:2, C36:1, C36:4, C38:1, C38:2, C38:3, C38:5, C38:6, C40:1, C40:2, C42:1, C42:2, C42:3), five lyso-phosphatidylcholines: lysoPC a (C24:0, C26:0, C26:1, C28:0, C28:1) as well as Met, Met-SO and the metabolic ratio Met-SO/Met (Figure 2).

Paired t tests with baseline-corrected data set revealed no significant concentration changes between the two sessions and PLS-DA showed no statistical significant separation (Figure 2).

DISCUSSION

Only limited information is available concerning the influence of acute or chronic drug of abuse consumption on the metabolome. Analysis of metabolomic changes after MDMA consumption was studied recently in rats indicating increased carnitine and decreased choline levels.[15] Nielsen et al. performed the first study with MDMA in humans with retrospective data from random forensic blood samples.[16] Unfortunately, controlled studies with application of drugs of abuses are pretty rare. In most countries, such studies with illicit substances are forbidden. In the rare cases where controlled studies on drugs of abuse are available, changes in metabolome are often not investigated.

Therefore, we aimed to analyze for the first time plasma samples from a highly controlled clinical study on MDMA to reveal metabolic changes. The MDMA dose administered can be regarded as relatively high compared to single, recreational MDMA doses.[13] Although the samples were stored over a period of up to 2.5 years and underwent a maximum of two freeze−thaw cycles, sufficient sample stability can be assumed based on previously published studies.[25,26] No evidence for decreasing or increasing concentration changes between the samples was discovered during the measurements or the evaluation of the data. The applied analytical kit has been validated for human blood plasma following FDA criteria[20] and generally allowed for detection and (semi-) quantification of 188 metabolites. However, 39 lipids were below the LOD, and missing data of some biogenic amines were discussed in a recently published interlaboratory comparison of the kit measurement.[27] Quality control samples were analyzed with each kit and showed acceptable variation (less than 20%) between plates and days. Nevertheless, it must be mentioned that not all metabolites are included in the commercial QC samples; mainly glycerophospholipids and spingolipids are missing.

Our data set had a multilevel structure which contained multiple types of variation, the within-individual variation, the between-individual variation or a combination thereof. The selection of important metabolites based on univariate statistical methods alone was not sufficient as metabolites often show nonsignificance in isolation but can result in reproducible discrimination in combination with other metabolites in a multivariate model. The classical multivariate analysis techniques PCA and PLS-DA were thus additionally applied to our data set. Ultimately, a combination of both univariate and multivariate analyses seemed best for detecting as many metabolite changes as possible. The different statistical methods showed similar results, which confirmed our findings. Multivariate analysis using PCA showed no clear clustering of the two sessions at any time point after the MDMA or placebo intake. PCA is a useful tool for the detection of biological processes but not ideal for biomarker discovery as there is no focus on finding differences between groups. PCA was however useful for detecting outliers. An outlier detected at time point 3 most likely caused by insufficient sample amount was then further excluded for PLS-DA analysis. PLS-DA can be superior to PCA as it attempts to highlight components that would separate the two sessions. It uses a different response for both sessions to decompose the data into latent variables to maximize covariance. The PLS-DA plot showed a relatively good separation between the two sessions at time point 2 and time point 3 of the uncorrected data set. However, application of PLS-DA on the corrected data set could not clearly separate the two session groups indicating that the interday variability was too high. Several metabolites, including Met-SO/Met and some glycerophospholipids, contributed considerably (VIP scores >1.65) to the model. They were also identified in univariate analyses, showing significantly concentration changes between the two sessions.

Many AA as well as taurine, creatinine, and kynurenine showed significant concentration changes between time points. However, a majority of these metabolites showed no significant effect in any other statistical test. Nielsen et al. discovered a downregulation of Trp in response to MDMA consumption with their retrospective untargeted metabolomics approach.[16] In our study, Trp was not appreciably altered between placebo and MDMA intake. With ANOVA analysis, we could only see significant changes in Trp concentrations between the different time points indicating rather time-dependence of Trp changes then MDMA influence. Circadian changes of certain AA have been studied intensively and thus we hypothesize these metabolites to change in a time-dependent manner independent of the MDMA intake.[28−30]

All tests showed statistically significant changes of the metabolic ratio Met-SO/Met indicating changes in systemic oxidative stress. Although Met itself did not meet the statistically significance level, p-values of Met for the baseline-corrected t tests were <0.05 for time point 1 and time point 2, respectively. Methionine functions as an important antioxidant defense mechanism. Various reactive oxygen species (ROS) react with Met to form Met-SO.[31] With a catalytic reaction by the repairing enzyme methionine sulfoxide reductases (Msr), Met-SO is transformed back to Met. Studies in mice showed that a lack of Msr resulted in enhanced sensitivity to oxidative stress and shorter lifespan. A high concentration of Met-SO is also correlated with various pathologic conditions in humans such as Alzheimer's disease or biological aging, and decreased levels of Met were found in psychotic affections.[32−34] This ratio showed significant differences with a VIP score >1.5 in PLS-DA analysis at time points 2 and 3. Similar changes of Met after MDMA consumption were reported elsewhere; Stuerenburg et al.[35] measured 33 AA in plasma samples of different ecstasy

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user groups. They reported that Met levels in the abstinent group differed significantly from two ecstasy user groups and a negative correlation with the cumulative dose and consumption in the last 12 months was found. MDMA is metabolized via N-demethylation to 3,4-methylenedioxy-amphetamine, and both are further demethylenated to catecholamines, which can undergo oxidation to quinones. These highly redox-active molecules can among other pathways produce semiquinone radicals leading to the generation of ROS. ROS productions are underlying processes of MDMA toxicity.[36−38] Although many other amino acids like Cys, His, Arg, Pro, Tyr, or Trp can undergo oxidative modifications, none of the others showed significant changes between the two sessions. No correlations between Met and Met-SO were found in the placebo (r = 0.03, time point 2) and the MDMA session (r = 0.01, time point 2) indicating that plasma concentrations of Met-SO are not results of spontaneous *in situ* oxidation reactions of Met during storage or sample processing.[39]

After MDMA intake, participants showed an increased amount of glycerophospholipids such as diacyl-phosphatidylcholines and acyl-alkyl phosphatidylcholines. This in general can be associated with changes in plasma lipoprotein, cell function, and inflammation and could be interpreted as an upregulation of energy production. The increased activity and cardiac stress after MDMA consumption cause an enhanced energy need that in part could be compensated by β-oxidation of fatty acids in muscle, heart, and liver.[13,15] No difference in concentration changes between shorter chained (≤C40) or longer chained (≥C42) PCs could be observed. The applied measurements cannot differentiate the fatty acids linked to the glycerol backbone resulting in a sum of structural isobaric and isometric lipids.[40] Only the total composition of lipid species was determined where side chain and substitution stereochemistry remains unknown.

Two lysoPCs (lysoPC a C26:1, lyso PC a C28:1) showed baseline-corrected significant concentration increases 8 h after MDMA intake. Lysophospholipids are hydrolyzed from phosphatidylcholines by the enzyme phospholipase A_2 and play an important role as intracellular messengers or can be further metabolized into mediators of a broad range of cellular processes. Nielsen et al. observed a downregulation of lysoPC species in response to MDMA suggesting decreased hydrolysis of lysoPC from phosphatidylcholine in membranes as a protection of mitochondrial membrane integrity.[16] However, we found a concentration increase of longer chained lysoPCs (lysoPC a C26:1 and lyso PC a C28:1) as compared to Nielsen et al., which is in our opinion conciliable with MDMA consumption. Lyso-phosphatidylcholine is a risk factor for vascular diseases. It is a major component of oxidized low-density lipoprotein and can increase the permeability of coronary artery endothelial cells in human.[41,42] MDMA as well as methamphetamine consumption is associated with an increased risk for various cardiovascular pathologies such as acute coronary syndrome, coronary artery disease, and acute aorta dissection.[13,43−45] Further research needs to be done to investigate if the detected lysoPCs might play a role in these pathologies.

The metabolite 5-HT, which plays an essential role in the pharmacological mechanism of MDMA, showed no significant changes in any statistical analyses of the different sessions and time points. Serotonin is stored in blood platelets that are prone to generate artificial increases due to damage and lysis during sample extraction making measurements of serotonin concentrations in blood difficult.[46]

The examined metabolites could be classified into several different metabolic pathways but did not allow to unambiguously identify pathways that would exclusively relate to MDMA consumption. A main reason could be that the metabolites targeted with the Biocrates kit are generally metabolites, which are involved in multiple pathways. Additionally, the body has several compensation mechanisms that can handle physiological stress situations that occur after a single intake of MDMA. Repeated intake of MDMA would possibly show more significant changes of these targeted metabolites but is more or less impossible to test under controlled conditions due to ethical restrictions. Targeted metabolomics measures only a defined set of metabolites, typically focusing on pathways of interest. These methods are usually highly specific and quantitatively reproducible what we were aiming for in a first step of our study. For a wider analysis of the changes in the human metabolome after intake of MDMA, an untargeted screening approach would be more suitable and is planned for future studies with these plasma samples. Although untargeted metabolomics data sets are highly complex and some metabolites remain often uncharacterized in structure and function, this approach allows searching for affected metabolites of for example inflammatory or hormonal pathways which would be of interest.

The identification of significant changes of metabolite concentrations primarily depends on the comparison between MDMA intake versus nonintake. The metabolome is however also sensitive to both internal (such as sex, age, and genetics) and external (such as diet, lifestyle, and analytical procedures) factors. Those factors can enhance or mask differences between plasma samples and therefore can be sources of bias.[17] The participants of this study were in the same age range (20−27 years, median 25 years), and gender was balanced, which reduced confounding factors. We tested if possible gender or age effects could have an effect on the data set (data not shown), but no significant influence of these factors has been seen in both sessions. Gender-specific differences in certain metabolites have been detected in previous large-scale studies.[47−49] The relatively small size of our study could be an explanation for not observing any gender- or age-specific effects on the metabolite concentrations. Furthermore, a lack of a suitable study design is often a problem in metabolomics studies. Certain specifications need to be fulfilled, for example, the experiment type, experimental variables (e.g., time dependence), control groups, number of replicates, and acceptance criteria. There are several metabolomics studies that lack zero/start point-samples for the comparison before and after treatment.[50,51] In our study, time points before the intake of MDMA as well as different time points in a placebo group were used and measured as reference data. A major strength of this study is the crossover design where the interventions are evaluated on the same participants, allowing a comparison at the individual level and reduction of variances. The metabolomics standards initiative (MSI) suggests a minimum of three to five replicates with a preference of biological replication over technical replication.[52] More participants would improve the statistical power of human metabolomic studies but are often impossible to perform due to costs, availability, or ethics. The primary aim of the original clinical study was to investigate the pharmacokinetic and pharmacodynamic effects of MDMA, and it lacked strict instructions

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regarding hours of sleep, activities, or fasting periods prior to the plasma-sampling day. By using baseline-corrected data, we aimed to overcome metabolic differences between the session days (interday individualities) of the participant. Statistical calculations between the two sessions at every time point showed that correcting for theses interday differences results in better separations with stronger outcome, especially with the PLS-DA calculations. Repeating the statistical tests with baseline-corrected metabolite concentrations showed no significant changes anymore with the multivariate analysis showing that a suitable "zero sample" is very important for the outcome of statistical calculations.

CONCLUSION

An overall increase in oxidative stress could be confirmed by increasing concentrations of the metabolic ratio Met-SO/Met. Many metabolites especially AA showed time dependent concentration changes rather unrelated to the MDMA intake. Baseline or zero samples within an experimental or observational study design are crucial for the evaluation of metabolomics data as the interday individuality within subjects was very high and false-positive results or an overestimation of the findings would be likely. A single intake of MDMA did not strongly alter the targeted metabolites. Unfortunately, the detected metabolites with significant concentration changes are not suited as possible biomarkers or biomarker pattern as they are rather unspecific and involved in many pathways in the human metabolome. A repeated or frequent intake of MDMA combined with untargeted analysis might improve monitoring of metabolome changes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00294.

List of metabolic ratios used for statistical analysis (PDF)

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Notes

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ABBREVIATIONS

MDMA, 3,4-methylenedioxymethamphetamine; DA, dopamine; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; LC, liquid chromatography; FIA, flow injection analysis; AA, amino acids; PC, phosphatidylcholines; IS, internal standard; MRM, multiple reaction monitoring; QC, quality control; M, MDMA session; P, placebo session; FDR, false discovery rate; ANOVA, analysis of variance; PCA, principal component analysis; PLS-DA, partial least-squares discriminant analysis; CMV, cross model validation; VIP, variable importance parameter; lysoPC, lyso-phosphatidylcholine; Met-SO, methionine sulfoxide; Met, methionine; Phe, phenylalanine; Thr, threonine; His, histidine; Tyr, tyrosine; Asn, asparagine; Arg, arginine; Leu, leucine; Ile, isoleucine; Lys, lysine; Asp, aspartic acid; Glu, glutamic acid; Trp, tryptophan; Val, valine; Gln, glutamine; Ala, alanine; tp, time point; Gly, glycine; ROS, reactive oxygen species; Msr, methionine sulfoxide reductase

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