Full-Length Original Research Article

Comprehensive study of metabolic changes induced by a ketogenic diet therapy using GC/MS- and LC/MS-based metabolomics

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Abstract

Objective: The ketogenic diet (KD), a high-fat and low-carbohydrate diet, is effective for a subset of patients with drug-resistant epilepsy, although the mechanisms of the KD have not been fully elucidated. The aims of this observational study were to investigate comprehensive short-term metabolomic changes induced by the KD and to explore candidate metabolites or pathways for potential new therapeutic targets.

Methods: Subjects included patients with intractable epilepsy who had undergone the KD therapy (the medium-chain triglyceride [MCT] KD or the modified Atkins diet using MCT oil). Plasma and urine samples were obtained before and at 2–4 weeks after initiation of the KD. Targeted metabolome analyses of these samples were performed using gas chromatography-tandem mass spectrometry (GC/MS/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS).

Results: Samples from 10 and 11 patients were analysed using GC/MS/MS and LC/MS/MS, respectively. The KD increased ketone bodies, various fatty acids, lipids, and their conjugates. In addition, levels of metabolites located upstream of acetyl-CoA and propionyl-CoA, including catabolites of branched-chain amino acids and structural analogues of γ-aminobutyric acid and lactic acid, were elevated.

Conclusions: The metabolites that were significantly changed after the initiation of the KD and related metabolites may be candidates for further studies for neuronal actions to develop new anti-seizure medications.

Keywords: amino acids; biomarkers; intractable epilepsy; ketone bodies; organic acids

Highlights

- Effects of the ketogenic diet on human plasma and urine metabolome were studied.
- Various metabolites upstream of acetyl-CoA and propionyl-CoA were elevated.
- Changed metabolites may hint toward developing new anti-seizure medications.

Introduction

Approximately 30% of patients with epilepsy have seizures that are refractory to anti-seizure medications (ASMs) [1–3]. The ketogenic diet (KD), a high-fat and low-carbohydrate diet, is effective for a subset of such patients [4,5]. Although the mechanism of the KD efficacy for epilepsy has not been fully elucidated, multiple mechanisms have been proposed and they may manifest anti-seizure effects in combination [6,7]. Recently, lactate dehydrogenase (LDH) inhibitors have been demonstrated to be a potential new target of ASMs based on theoretical considerations of metabolic changes induced by the KD, raising the possibility of targeting cellular metabolism in new ASM development [8]. Other unknown mechanisms suppressing epileptic seizures may still exist because the metabolic changes induced by the KD would be broad.

With the advent of mass spectrometry-based technology, comprehensive metabolic changes induced by the KD can be investigated using metabolome analysis. However, so far, there have been few numbers of such studies on animals [9–11] and humans [12]. This previous human study included paediatric epilepsy patients who had undergone the KD therapy and analysed the cerebrospinal fluid (CSF) metabolome [12], yet the analysis of metabolites focused on only lipids and carbohydrates. This study also required a lumbar puncture, which is not readily feasible in regular clinical settings.

In this multicentre study, we conducted gas chromatography-mass spectrometry (GC/MS)-based and liquid chromatography-mass spectrometry (LC/MS)-based metabolome analyses of plasma and urine samples obtained from paediatric patients who had drug-resistant epilepsy who had undergone the KD therapy. We aimed to demonstrate comprehensive metabolic changes induced by the KD therapy and explore candidate metabolites or pathways for potential new ASM targets.

Materials and methods

Study protocols

This research was conducted as a multicentre observational study in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Okayama University Hospital (approval #1604-009). Written informed consent was obtained from the parents or guardians of all patients. We enrolled patients with drug-resistant epilepsy who had received the KD therapy for at least 3 months at Okayama University Hospital or other collaborative hospitals between December 2018 and June 2021. There were no strict restrictions on changes in other medications and co-administration of supplements. The plasma and urine samples were obtained at baseline (prior to the KD therapy) and 2–4 weeks after the initiation of the KD in the early morning during hospitalization, when there were opportunities to perform blood and urine tests to monitor ketone bodies and check for adverse effects.

Sample handling protocols are shown in Supplementary Fig. 1. Blood samples were collected into a test tube containing ethylenediaminetetraacetic acid disodium salt (PN5 test tube, SRL, Tokyo, Japan) and cooled on ice immediately. Samples were centrifuged at 2000 × *g* at 4°C for 20 minutes within 2 hours of collection. The separated plasma was aliquoted and frozen within 4 hours of collection. If the freezing temperature was not below −70°C, the frozen samples were transferred to a deep freezer within 8 hours of collection. Urine samples were immediately cooled on ice after collection, and were centrifuged at 2000–3000 × *g* at 4°C for 5 minutes within 2 hours of collection. The supernatant was aliquoted and frozen at −70°C or lower in the same manner as for the plasma samples.

Targeted metabolome analysis

All samples were collected at Okayama University Hospital and shipped to Tohoku Medical Megabank Organization at Tohoku University on dry ice. Targeted metabolic profiling was conducted using gas chromatography-tandem mass spectrometry (GC/MS/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). GC/MS/MS analysis was performed according to a previously reported method, using a GC-MS TQ8040 system (Shimadzu, Kyoto, Japan) [13,14]. The database used in this study includes data on 475 peaks from 333 metabolites.

LC/MS/MS analysis was performed using ACQUITY UPLC I-Class and Xevo TQ-S systems (Waters, Milford, USA). The MxP® Quant 500 kit (Biocrates, Innsbruck, Austria) was used as a measurement kit, and targets up to 630 metabolites from 26 biochemical classes.

Statistical analysis

Statistical analysis was performed on the measured plasma and urine metabolite concentration data using MetaboAnalyst 5.0 analysis (https://www.metaboanalyst.ca/). Metabolites with missing values (e.g., below the limit of quantification) were excluded. In the urine sample, the metabolite concentration was corrected using the creatinine concentration. The concentration data were transformed logarithmically to increase normality and scaled so that the mean value was 0 and the variance was 1 for each metabolite.

Differences between the two groups were examined using univariate and multivariate analyses. Univariate tests included Wilcoxon signed-rank test for paired data and Mann–Whitney *U*-test for unpaired data, considering multiple comparisons. A metabolite with a false discovery rate (FDR) <0.05 was determined to be a metabolite with a significant change between the two groups. Next, partial least squares discriminant analysis (PLS-DA), a multivariate analysis, was performed to determine the metabolites with a high degree of contribution to the group difference. Model performance was evaluated by cross-validation using calculated R2 (goodness of fit) and Q2 (goodness of prediction) values. A Q2 value ≥ 0.5 was considered a good prediction using a constructed model [15]. In this case, a metabolite with variable importance for prediction (VIP) score ≥ 1.5 was determined to be a metabolite with a significant change between the two groups.

Results

Clinical profiles of subjects

We analysed plasma and urine samples from 11 patients obtained at baseline and at 2–4 weeks after initiation of the KD (Supplementary Table 1). Although the fasting time prior to sample collection was more than 4 hours in most samples, a few samples had to be obtained at 2–3 hours

after the last meal. The age at starting the KD ranged from 8 months to 17 years 3 months (median, 3 years 11 months). The type of epilepsy included West syndrome in five cases, Dravet syndrome in three cases, generalized epilepsy (not belonging to a specific epilepsy syndrome) in one case, Lennox–Gastaut syndrome in one case, and focal epilepsy (not belonging to a specific epilepsy syndrome) in one case. Seven patients underwent the medium-chain triglyceride (MCT) KD and three patients underwent the modified Atkins diet. A ketogenic formula (Ketone Formula, Meiji, Tokyo) containing 71.8% (w/w) of lipids (39.7% [w/w] of MCT oil) was used concurrently for both types of diets. The ketogenic ratio for the MCT KD ranged from 1.5:1 to 3:1, and the daily amount of carbohydrates of the modified Atkins diet ranged from 10 to 15 g/day. The seizure reduction rate at 3 months after the start of the KD from baseline (4 weeks before the start of the KD) was markedly effective (≥75%) in two cases, effective (50–75%) in six cases, and ineffective (<50%) in three cases. There were no cases of complete seizure suppression at that time. The KD therapy had been discontinued by 5 months after the start of the KD in three ineffective cases (cases #9, #10, and #11). Other cases had continued the KD therapy for up to 4 years 2 months at the last follow-up. Two cases (cases #1 and #6) had to discontinue the KD because of difficulty in maintaining the KD or its side effects. One case (case #5) tapered off the KD due to loss of effect. One case (case #4) continued the KD despite reduced effect. The KD therapy remained effective in others (cases #2, #3, #7, and #8) at the last follow-up.

Plasma metabolome changes induced by the KD

Paired samples before and 2–4 weeks after the start of the KD were analysed to investigate the short-term metabolic changes induced by the KD. We analysed 20 (10 paired) plasma samples and 20 (10 paired) urine samples from 10 patients using LC/MS/MS. After plasma and urine samples from one additional case (case #8) were obtained, we analysed 22 (11 paired) plasma samples and 22 (11 paired) urine samples from 11 cases using GC/MS/MS.

The plasma GC/MS/MS data demonstrated that 11 of the quantified 165 metabolites showed significant changes using a univariate test (Wilcoxon signed-rank test, FDR <0.05) (Table 1). Of

these 11 metabolites, 5 metabolites (3-hydroxybutyric acid, acetoacetic acid, 3-hydroxyisobutyric acid, acetylglycine, and 2-hydroxybutyric acid) showed a significant increase to more than double, and two metabolites (glyoxylic acid and cystine) showed a significant decrease to less than half (Fig. 1A). Multivariate analysis (PLS-DA) (three components, R2 = 0.96, Q2 = 0.75) demonstrated an additional five elevated metabolites (decanoic acid, octanoic acid, isoleucine, adipic acid, and uric acid) and four reduced metabolites (glucosamine, *N*-acetyllysine, galactose, and mannitol) contributing to group difference (VIP score ≥ 1.5) (Table 1, Fig. 1B).

The plasma LC/MS/MS data demonstrated that 45 of the quantified 329 metabolites showed significant changes using a univariate test (Wilcoxon signed-rank test, FDR <0.05) (Table 2). PLS-DA (three components, R2 = 0.96, Q2 = 0.67) demonstrated that 46 metabolites contributed to group difference (Table 2). The metabolites that were significantly increased were mostly lipids such as triglycerides and diglycerides, and acylcarnitines (C2 [acetylcarnitine], C4-OH [3-hydroxybutyrylcarnitine], and C18 [stearoylcarnitine]). In addition, an increase in 2-aminobutyric acid and 3-aminobutyric acid, and a decrease in cystine, cysteine, and homoarginine were observed.

Urine metabolome changes induced by the KD

The urine GC/MS/MS data demonstrated that seven of the quantified 175 metabolites showed significant changes using a univariate test (Wilcoxon signed-rank test, FDR <0.05) (Table 3). Of these seven metabolites, six metabolites showed a significant increase of more than double (Fig. 2A). The increased metabolites included 3-aminoisobutyric acid and 3-hydroxypropionic acid, in addition to those significantly increased in plasma. PLS-DA (eight components, R2 = 1.00, Q2 = 0.65) demonstrated six additional elevated metabolites (2-aminobutyric acid, sebacic acid, glyceric acid, 3-hydroxy-3-methylglutaric acid, suberic acid, and 3-hydroxyisovaleric acid) and eight reduced metabolites (catechol, tagatose, 2-aminopimelic acid, citric acid, mannitol, glucose, tartaric acid, and 3-hydroxyanthranilic acid) contributing to group difference (Table 3, Fig. 2B).

The urine LC/MS/MS data showed that no metabolite was significantly altered among the 65 quantified metabolites using a univariate test. PLD-DA (8 components, R2 = 1.0, Q2=0.50) demonstrated five metabolites contributing to group difference (Supplementary Table 2). The increased metabolites included 3-aminobutyric acid, an acylcarnitine (C5:1-DC [glutaconylcarnitine]), and lipids (hexosylceramides).

Discussion

We conducted a metabolomics study to investigate the comprehensive metabolite changes induced by the KD in humans and explored metabolites associated with the short-term efficacy of the KD therapy. The combination of the GC/MS- and LC/MS-based metabolome analyses succeeded in the detection of metabolite changes between pre-treatment samples and those obtained at 2–4 weeks after the initiation of the KD therapy. The metabolome changes involved various pathways beyond ketone body synthesis and fatty acid oxidation, especially the degradation of branched-chain amino acids (valine, leucine, and isoleucine) (Fig. 3).

Increases in the ketone bodies (3-hydroxybutyric acid and acetoacetic acid), and various fatty acids and lipids are regarded as direct changes caused by the KD, including a metabolic shift from glucose-based to fat-based energy generation and increased lipogenesis. Among them, decanoic acid (a medium-chain fatty acid) has been shown to reduce seizures via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor [16], and this anti-seizure effect has been enhanced by the presence of octanoic acid, another medium-chain fatty acid [17,18]. An increase in these medium-chain fatty acids is likely to be due to the use of MCT oil in our patients.

Elevation of acetylglycine and C2 (acetylcarnitine) suggests an increase in acetyl-CoA, which is the product of enhanced β-oxidation by a large supply of fats under a low-carbohydrate condition. An excess of acetyl-CoA led to the production of ketone bodies via acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA. Elevations of C4-OH (3-hydroxybutyrylcarnitine) and 3-hydroxy-3-methylglutaric acid are likely to be due to increases in the levels of 3-hydroxybutyric

acid and 3-hydroxy-3-methylglutaryl-CoA, respectively. Furthermore, an increase in 3-hydroxyisovaleric acid was observed. In the metabolism of leucine, which is a ketogenic amino acid, the elevation of the intermediate metabolite 3-hydroxy-3-methylglutaryl-CoA led to an increase in the immediate upstream metabolite, 3-methylcrotonyl-CoA. Excess 3-methylcrotonyl-CoA entered an alternative metabolic pathway to produce 3-hydroxyisovaleric acid via 3-hydroxyisovaleryl-CoA. Elevation of 3-hydroxyisovaleric acid in ketoacidosis has been reported previously [19].

An increase in 3-hydroxypropionic acid suggests an increase in propionyl-CoA, resulting from enhanced β-oxidation of odd-chain fatty acids. 3-Hydroxypropionic acid is a well-known biomarker of propionic acidaemia that causes the accumulation of propionyl-CoA. Increases in 3-hydroxyisobutyric acid and 3-aminoisobutyric acid, which are intermediate metabolites of valine and thymine, respectively, are also likely to be due to the increase in propionyl-CoA. The elevation of isoleucine, which is metabolized to propionyl-CoA and acetyl-CoA, can be explained similarly. Increases in 2-hydroxybutyric acid and 2-aminobutyric acid levels are also probably caused by elevated propionyl-CoA levels. These are interconverted via 2-ketobutyric acid. However, this study demonstrated that urine 2-ketobutyric acid was not significantly changed by the KD, and plasma 2-ketobutyric acid was below the low limit of quantification. In the physiological state, 2-ketobutyric acid enters the tricarboxylic acid cycle via propionyl-CoA. When this conversion is insufficient, it is probably metabolized rapidly to 2-hydroxybutyric acid or 2-aminobutyric acid.

Among other increased metabolites, adipic acid, suberic acid, and sebacic acid are straight-chain, saturated dicarboxylic acids. The elevation of these acids has been reported in ketoacidosis caused by fasting and diabetes [20]. They are produced by ω-oxidation and subsequent β-oxidation of long-chain monocarboxylic acids. Glycerol 3-phosphate and glyceric acid are generated via the phosphorylation and oxidation of glycerol, respectively. The elevation of these compounds is likely to be due to an increased supply of glycerol, which is an important

component of triglycerides in the KD. Elevation of plasma uric acid, which is a well-known side effect of the KD, is explained by the reduction in its renal excretion caused by the ketone bodies [21]. An increase in 3-aminobutyric acid levels was detected in plasma and urine using LC/MS/MS. Although there have been reports on its role in plants, we could not find the biological significance of this amino acid in human metabolic pathways.

Investigation of the neuronal actions of these elevated metabolites and related metabolites may help to understand the anti-seizure effects of the KD. Among them, 2-aminobutyric acid and 3-aminobutyric acid are isomers of γ-aminobutyric acid (4-aminobutyric acid), an inhibitory neurotransmitter. 2-Hydroxybutyric acid is a structural analogue of lactic acid (2-hydroxypropionic acid) and it is converted to 2-ketobytyric acid (an analogue of pyruvic acid [2-ketopropionic acid]) and vice versa by LDH, a new target enzyme for ASM development based on the KD [8]. It is of interest that 2-ketobutyric acid has been reported to suppress excitatory postsynaptic currents [22]. Although we did not find any significant alteration in this metabolite in plasma and urine, it would be interesting in future studies to investigate its intracellular levels.

Cystine is of interest among metabolites reduced by the KD, because its metabolism shares 2-ketobutyric acid as described previously, and it is also involved in the synthesis of glutathione, an antioxidant. The relevance of the glutathione system has been proposed in epilepsy [2,24], and the KD has been shown to increase glutathione in the brain [25]. Reduction in cystine, a dimer of cysteine, suggests a decrease in the cysteine/cystine pool. Cysteine and 2-ketobutyric acid are produced from cystathionine. The synthesis of glutathione from cysteine is facilitated by 2-aminobutyric acid produced from 2-ketobutyratic acid [26]. Conversely, the synthesis of cysteine from cystathionine is inhibited by acetoacetic acid, a ketone body [27]. Therefore, further study is required to clarify whether the reduction in the cysteine/cystine pool reflects an overconsumption of cysteine caused by the enhanced synthesis of glutathione or a reduced supply of cysteine caused by ketone bodies.

There are several limitations to this study. The sample size was small and we were unable to explore metabolites correlated with the KD efficacy. Plasma and urine samples do not directly reflect metabolic status in the brain, although these samples can be collected less invasively than from the CSF. As this study was an observational study, we were unable to control the co-administration of ASMs and supplements, and the conditions (e.g., fasting time) for sample collection. Future directions include the investigation of longer-term metabolic changes after the initiation of the KD. We are also planning to increase the number of cases to obtain more robust results on metabolic changes induced by the KD and to detect metabolites that contribute to predicting the efficacy of the KD before or at the early stages of treatment.

Conclusions

We demonstrated short-term (2–4 weeks) metabolic changes in plasma and urine samples induced by the KD and obtained from patients who had drug-resistant epilepsy using GS/MS- and LC/MS-based metabolome analyses. In addition to ketone bodies, fatty acids, lipids, and their various conjugates, the KD increased metabolites located upstream of acetyl-CoA and propionyl-CoA, including catabolites of branched-chain amino acids and structural analogues of γ-aminobutyric acid and lactic acid. These metabolites and related metabolites may be candidates for further studies for neuronal actions to develop new ASMs.

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Declarations of interest

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Table 1. GC/MS/MS results for plasma metabolite changes by the ketogenic diet therapy

Metabolites	Univariate test FDR	Multivariate test VIP score	Fold change
Increased			
3-Hydroxybutyric acid	**0.027**	**2.81**	**15.2**
Acetoacetic acid	**0.027**	**3.09**	**17.7**
3-Hydroxyisobutyric acid	**0.027**	**2.97**	**19.5**
Acetylglycine	**0.027**	**2.45**	**2.7**
2-Hydroxybutyric acid	**0.036**	**2.25**	**2.2**
Citramalic acid	**0.044**	**1.91**	1.7
Decanoic acid	0.051	**2.52**	**3.2**
Octanoic acid	0.051	**2.04**	**5.5**
Isoleucine	0.077	**1.85**	1.7
Adipic acid	0.052	**1.72**	**2.5**
Uric acid	0.116	**1.68**	1.5
Decreased			
Glyoxylic acid	**0.027**	**1.89**	**0.49**
Psicose	**0.027**	1.20	0.75
Cystine	**0.036**	**1.82**	**0.50**
2′-Deoxyuridine	**0.036**	0.82	0.78
Hippuric acid	**0.044**	**2.07**	0.86
Glucosamine	0.051	**1.95**	0.85
N-Acetyllysine	0.077	**1.81**	0.84
Galactose	0.077	**1.74**	0.85
Mannitol	0.077	**1.72**	0.86

FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; VIP, variable importance for prediction.

FDRs <0.05, VIP scores ≥1.5, and fold changes ≤0.5 or ≥2.0 are indicated in bold.

Table 2. LC/MS/MS results for plasma metabolite changes by the ketogenic diet therapy

Metabolites	Univariate test FDR	Multivariate test VIP score	Fold change
Increased			
Glutamic acid	**0.022**	1.25	1.5
2-Aminobutyric acid	**0.022**	**1.74**	1.9
C2	**0.022**	**2.17**	**3.1**
C4-OH (C3-DC)	**0.022**	**2.70**	**6.8**
DG (16:0_18:2)	**0.022**	**1.75**	**2.6**
DG (18:2_18:2)	**0.022**	**1.73**	**2.5**
TG (16:0_36:3)	**0.022**	1.44	1.9
TG (16:0_36:4)	**0.022**	**1.65**	**2.4**
TG (18:0_36:2)	**0.022**	**1.85**	**2.8**
TG (18:0_36:3)	**0.022**	**2.02**	**3.1**
TG (18:1_36:1)	**0.022**	**1.52**	**2.2**
TG (18:1_36:3)	**0.022**	**1.52**	**2.2**
TG (18:1_36:4)	**0.022**	**1.62**	**2.4**
TG (18:2_34:0)	**0.022**	**1.64**	**2.3**
TG (18:2_34:1)	**0.022**	1.47	1.9
TG (18:2_34:2)	**0.022**	**1.60**	**2.3**
TG (18:2_36:1)	**0.022**	**1.96**	**2.8**
TG (18:2_36:2)	**0.022**	**1.66**	**2.4**
TG (18:2_36:3)	**0.022**	**1.82**	**3.0**
TG (20:4_36:3)	**0.022**	**1.89**	**2.3**
TG (20:4_36:4)	**0.022**	**1.80**	**2.4**
3-Aminobutyric acid	**0.034**	**2.26**	**2.3**
C18	**0.034**	**1.94**	**2.3**
DG (18:1_18:2)	**0.034**	1.39	1.9
Cer (d18:0/24:0)	**0.034**	**1.76**	**2.2**
Cer (d18:0/24:1)	**0.034**	**1.91**	**2.2**
TG (18:2_38:5)	**0.034**	**1.57**	1.9
TG (20:0_32:4)	**0.034**	**1.74**	**2.4**
TG (20:4_34:0)	**0.034**	1.17	1.9
C16-OH	**0.043**	1.26	1.7
C18:2	**0.043**	**1.51**	1.7
TG (18:2_32:0)	**0.043**	1.47	**2.3**
TG (20:4_34:2)	**0.043**	1.32	1.8
TG (20:4_36:2)	**0.043**	1.46	1.8
TG (17:2_38:6)	**0.043**	**1.56**	**2.4**
C4:1	0.061	**1.99**	1.6
Betaine	0.061	**1.94**	**2.1**
TG (22:0_32:4)	0.061	**1.81**	**2.3**
FA (20:2)	0.061	**1.75**	**2.1**
Isoleucine	0.123	**1.67**	1.6
TG (20:2_36:5)	0.113	**1.56**	1.5
SM C24:0	0.167	**1.55**	1.4
SM C26:0	0.123	**1.52**	1.4
PC aa C34:2	0.193	**1.51**	1.1
Decreased			
Cysteine	**0.022**	**2.11**	0.62
Cystine	**0.022**	**2.17**	0.58
Homoarginine	**0.022**	**1.62**	0.51
Cer (d18:2/20:0)	**0.022**	**1.69**	0.62

Cer (d18:2/24:0)	**0.022**	**1.74**	0.61
Cer (d18:2/24:1)	**0.022**	**2.08**	0.58
LysoPC a C16:1	**0.022**	**1.83**	**0.50**
LysoPC a C18:1	**0.022**	1.42	0.64
HexCer (d18:2/24:0)	**0.034**	1.41	0.74
LysoPC a C20:3	**0.043**	**2.02**	0.53
PC aa C38:3	0.061	**1.67**	0.66
Histidine	0.151	**1.63**	0.85
PC aa C32:1	0.061	**1.54**	0.66

C2, acetylcarnitine; C3-DC, malonylcarnitine; C4:1, butenoylcarnitine; C4-OH, 3-hydroxybutyrylcarnitine; C16-OH, 3-hydroxypalmitoylcarnitine; C18, stearoylcarnitine; C18:2, linoleoylcarnitine; C18-OH, 3-hydroxystearoylcarnitine; Cer, ceramide; DG, diglyceride; FA, fatty acid; FDR, false discovery rate; HexCer, hexosylceramide; LC/MS/MS, liquid chromatography-tandem mass spectrometry; LysoPC a, lysophosphatidylcholine acyl; PC aa, phosphatidylcholine diacyl; SM, sphingomyelin; TG, triglyceride; VIP, variable importance for prediction.

FDRs <0.05, VIP scores ≥1.5, and fold changes ≤0.5 or ≥2.0 are indicated in bold.

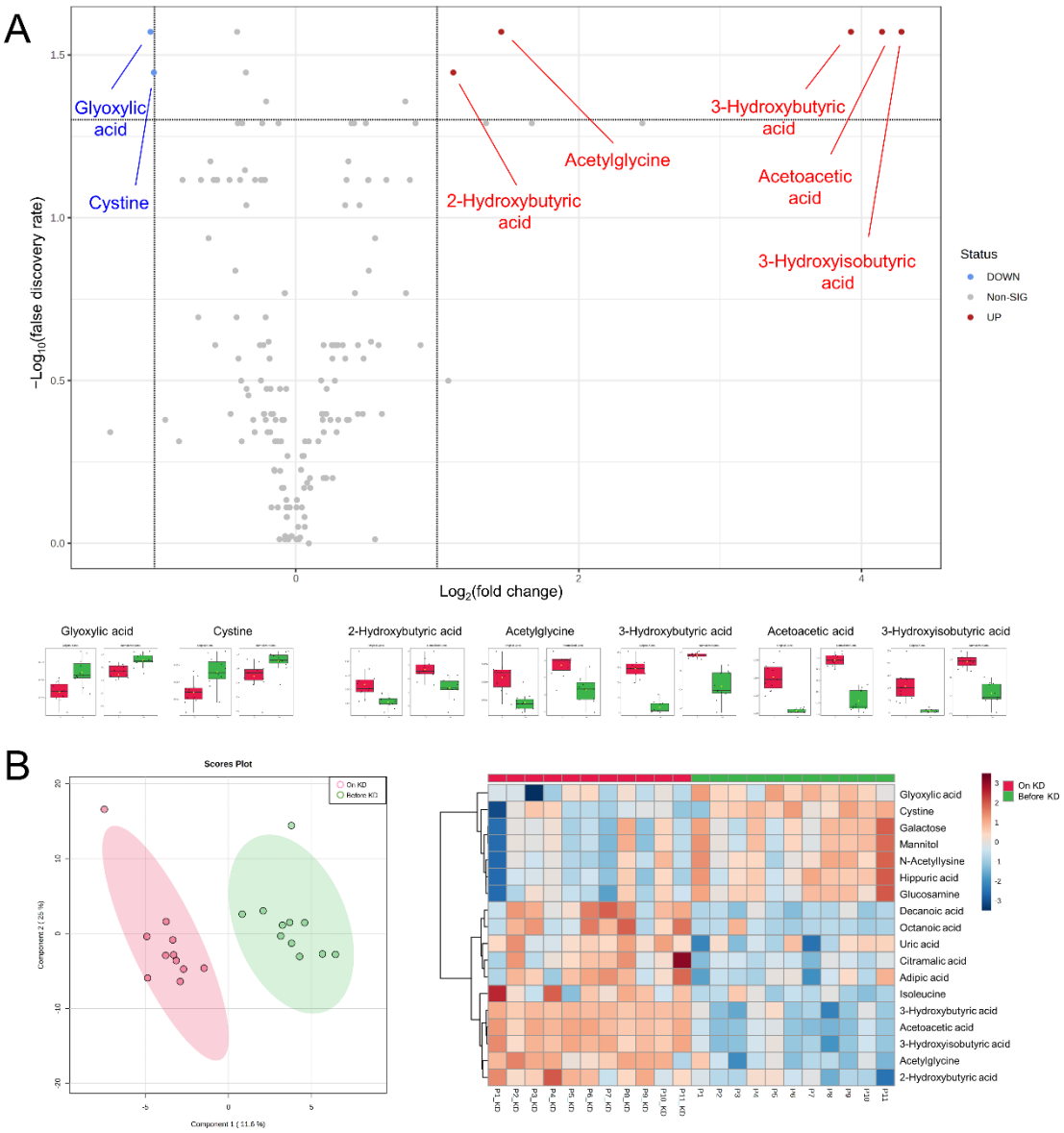
Table 3. GC/MS/MS results for urine metabolite changes by the ketogenic diet therapy

Metabolites	Univariate test FDR	Multivariate test VIP score	Fold change
Increased			
3-Hydroxybutyric acid	**0.043**	**3.51**	**139.5**
3-Hydroxyisobutyric acid	**0.043**	**3.28**	**33.4**
Acetylglycine	**0.043**	**2.90**	**9.7**
2-Hydroxybutyric acid	**0.043**	**2.89**	**10.6**
3-Aminoisobutyric acid	**0.049**	**1.96**	**3.9**
3-Hydroxypropionic acid	**0.049**	**1.92**	**2.9**
Glycerol 3-phosphate	**0.049**	1.22	1.9
2-Aminobutyric acid	0.313	**1.89**	**2.4**
Sebacic acid	0.323	**1.82**	**11.9**
Glyceric acid	0.155	**1.82**	**2.1**
3-Hydroxy-3-methylglutaric acid	0.066	**1.79**	**2.2**
Suberic acid	0.355	**1.67**	**4.4**
3-Hydroxyisovaleric acid	0.066	**1.52**	1.7
Decreased			
Catechol	0.066	**2.53**	**0.11**
Tagatose	0.085	**2.28**	**0.29**
2-Aminopimelic acid	0.107	**2.16**	**0.35**
Citric acid	0.141	**2.07**	**0.40**
Mannitol	0.155	**1.92**	**0.14**
Glucose	0.155	**1.90**	**0.14**
Tartaric acid	0.323	**1.51**	**0.37**
3-Hydroxyanthranilic acid	0.323	**1.50**	**0.24**

FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; VIP, variable importance for prediction.

FDRs <0.05, VIP scores ≥1.5, and fold changes ≤0.5 or ≥2.0 are indicated in bold.

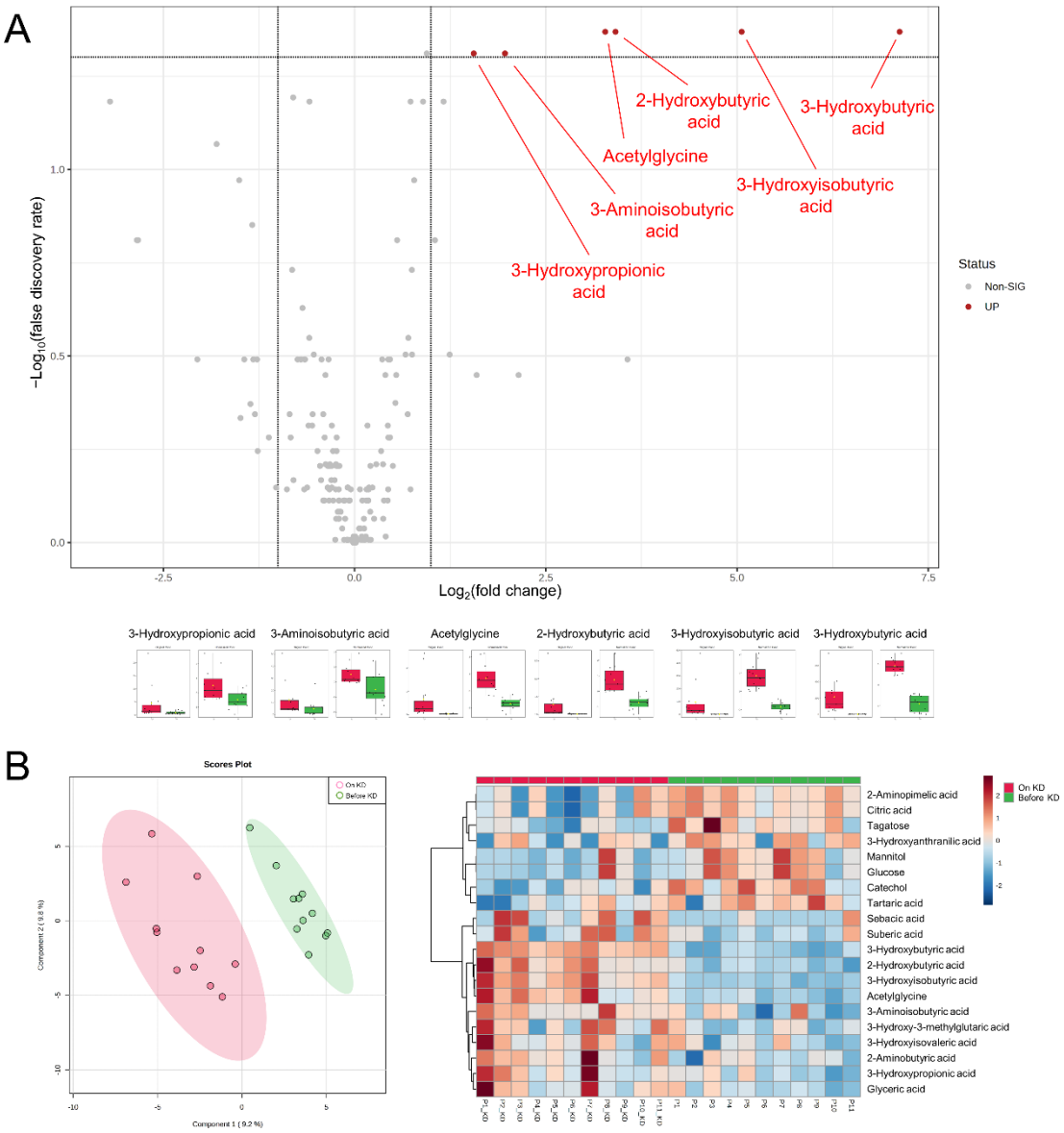
Figure 1. Statistical analysis for plasma GC/MS/MS data



A: Volcano plot showing fold change (horizontal axis) and FDR (vertical axis). Metabolites with a fold change of more than double or less than half, and with FDR <0.05, are highlighted. Their original and normalized concentrations are demonstrated below. B: Left: Score plot of first two components calculated using partial least squares discriminant analysis. Cross-validation demonstrated that the best model performance was achieved using three components (R2 = 0.96, Q2 = 0.75). Right: Heatmap showing the concentration of the metabolites with variable importance for prediction score ≥ 1.5.

FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; KD, ketogenic diet.

Figure 2. Statistical analysis for urine GC/MS/MS data

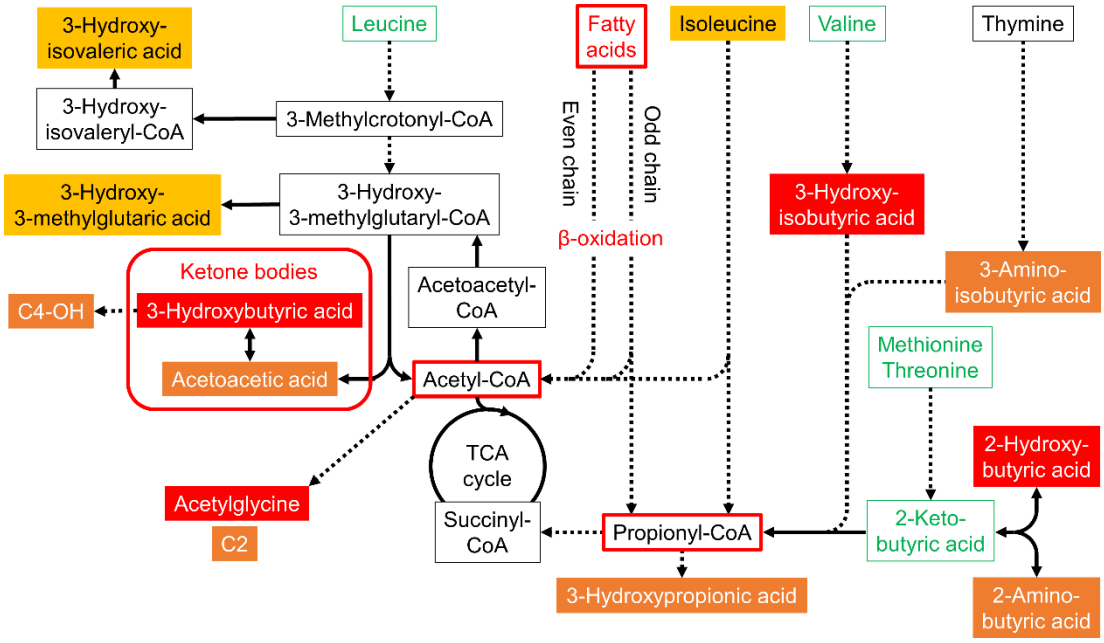


A: Volcano plot showing fold change (horizontal axis) and FDR (vertical axis). Metabolites with a fold change of more than double or less than half, and with FDR <0.05, are highlighted. Their original and normalized concentrations are demonstrated below. B: Left: Score plot of first two components calculated using partial least squares discriminant analysis. Cross-validation demonstrated that the best model performance was achieved using eight components (R2 = 1.00, Q2 = 0.65). Right: Heatmap showing the concentration of the metabolites with variable importance for prediction score ≥ 1.5.

FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; KD,

ketogenic diet.

Figure 3. Metabolites increased by the ketogenic diet and their related pathways



Metabolites filled with red were elevated in plasma and urine using univariate and multivariate analyses. Those filled with dark orange were elevated in plasma or urine using univariate and multivariate analyses. Those filled with light orange were elevated in plasma or urine using univariate or multivariate analysis. Those filled with green were not altered by the ketogenic diet. Those filled with black were not measured.

C2, acetylcarnitine; C4-OH, 3-hydroxybutyrylcarnitine; TCA, tricarboxylic acid.