



¹H NMR metabolomic and transcriptomic analyses reveal urinary metabolites as biomarker candidates in response to protein undernutrition in adult rats

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Abstract

Protein undernutrition contributes to the development of various diseases in broad generations. Urinary metabolites may serve as non-invasive biomarkers of protein undernutrition; however, this requires further investigation. We aimed to identify novel urinary metabolites as biomarker candidates responsive to protein undernutrition. Adult rats were fed control (CT; 14 % casein) or isoenergetic low-protein (LP; 5 % casein) diets for 4 weeks. ¹H NMR metabolomics was applied to urine, plasma and liver samples to identify metabolites responsive to protein undernutrition. Liver samples were subjected to mRNA microarray and quantitative PCR analyses to elucidate the mechanisms causing fluctuations in identified metabolites. Urinary taurine levels were significantly lower in the LP group than in the CT group at week 1 and remained constant until week 4. Hepatic taurine level and gene expression level of cysteine dioxygenase type 1 were also significantly lower in the LP group than in the CT group. Urinary trimethylamine N-oxide (TMAO) levels were significantly higher in the LP group than in the CT group at week 2 and remained constant until week 4. Hepatic TMAO level and gene expression levels of flavin-containing mono-oxygenase 1 and 5 were also significantly higher in the LP group than in the CT group. In conclusion, urinary taurine and TMAO levels substantially responded to protein undernutrition. Furthermore, changes in hepatic levels of these metabolites and gene expressions associated with their metabolic pathways were also reflected in their fluctuating urinary levels. Thus, taurine and TMAO could act as non-invasive urinary biomarker candidates to detect protein undernutrition.

Key words: Metabolomics: Multivariate analysis: Protein undernutrition: Transcriptomics: Urinary metabolites

It is estimated that an additional 50 % protein will be required by 2050 as the global population increases⁽¹⁾, which may put individuals from all generations at an increased risk of protein undernutrition. Several studies have also shown that inadequate protein intake in broad generations, including younger individuals, might lead to reduced physical activity through the effect on muscles^(2,3). In addition, it is speculated that the reduced physical activity in individuals from younger generation would increase the risk of disease development later in life. Besides, several studies have reported that protein undernutrition may induce fatty liver and lead to liver disease in the long term^(4–7). Thus,

because protein undernutrition has potential adverse effects on living tissues such as the muscles and liver, it would be desirable to establish minimally invasive and easy-to-collect biomarkers detecting the nutritional status as early as possible. Biomarkers that reflect protein undernutrition in bio-fluid samples such as blood and urine could be extremely useful for the prevention of chronic protein malnutrition and the secondary diseases in a wide generation range.

The levels of specific circulating proteins such as transthyretin, transferrin and albumin in blood have been reported to correlate with protein nutritional status^(8,9). Furthermore, it has been

Abbreviations: ASL, argininosuccinate lyase; ASS1, argininosuccinate synthase 1; CDO1, cysteine dioxygenase type 1; CT, control; CV-ANOVA, cross-validated ANOVA; FMO, flavin-containing mono-oxygenase; GO, gene ontology; LP, low-protein; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; ppm, parts per million; TCA, tricarboxylic acid; TMAO, trimethylamine N-oxide; TSP, 3-(trimethylsilyl)propionic acid-d4 sodium salt.

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reported that urine as well as blood may be able to detect protein nutritional status⁽⁹⁾. Urine could be advantageous over blood, as the sampling is non-invasive and easy to perform frequently. However, unlike proteins in blood, those in urine are difficult to apply widely as a biomarker because they are only detected in individuals with renal dysfunction⁽¹⁰⁾. Hence, low-molecular-weight metabolites, non-protein components in urine, are generally focused on. Although urinary nitrogen has been reported as the metabolite that correlates with total protein intake, nitrogen sources other than protein make a greater contribution to total nitrogen levels⁽¹¹⁾. 3-Methylhistidine is a urinary metabolite that correlates with protein nutritional status; however, the excretion level is reported to increase with meat intake^(12,13), and the nutritional status in response to meat-derived proteins may be overestimated. Thus, to the best of our knowledge, there are few suitable urinary metabolites that correlate with protein nutritional status.

Metabolomic approaches provide low-molecular-weight metabolite profiles to help understand biological responses. Highly informative metabolic profiles can be a potential source of biomarkers; therefore, metabolomics has been a promising tool for the diagnosis of several diseases such as cancer and heart diseases^(14,15). Metabolomics can also be used for the discriminant analysis of physiological responses to nutritional status⁽¹⁶⁾. NMR and MS have been used as techniques for metabolomics. The NMR method is less sensitive; however, it has many advantages such as high reproducibility, high quantitation, minimal sample requirement and non-destructive characteristic compared with MS methods⁽¹⁷⁾. Therefore, the NMR analysis was applied to identify and quantify complex metabolites in biological samples such as urine, plasma and liver in this study.

We have recently investigated the effects of moderate protein undernutrition on plasma protein profiles in adult Wistar rats where rats were maintained on an AIN-93 M (14% casein, control (CT) diet) or AIN-93 M-based isoenergetic diet containing 5% casein (low-protein (LP) diet) for 4 weeks⁽¹⁸⁾. The use of this animal model has been justified by confirming that this nutritional condition reflects the potential protein undernutrition in human⁽¹⁹⁾. Here, we expanded the investigation on the same animals, focusing on their metabolites. The aim of this study was to identify novel urinary metabolites as biomarker candidates that were responsive to protein undernutrition using NMR metabolomic and transcriptomic analyses.

Experimental methods

Animal experiments

The study design was approved by the Animal Research Committee of Morinaga Milk Industry Co. Ltd (approval number: 18-025), and experiments were performed in accordance with their guidelines (27 September–9 November 2018). The detail has already been described in our recent report⁽¹⁸⁾. Briefly, twelve male Wistar rats (14 weeks old; Japan SLC) were acclimated individually to polycarbonate cages with wood shavings in temperature-, humidity- and light-controlled facility (21–25°C,

40–60% and a 12 h light–12 h dark cycle) by allowing them *ad libitum* access to water and the CT diet for 2 weeks. Then, animals were assigned to two dietary groups, being fed *ad libitum* either the CT diet or the LP diet for 4 weeks (respectively, *n* 6). This sample size is the minimum necessary for reliable statistical verification. Urine samples were obtained weekly during the experimental period using metabolic cages. At the experimental period (after 4 weeks), animals were euthanised by deep anaesthesia with sevoflurane and blood samples were drawn from the inferior vena cava using syringes. Blood samples were centrifuged at 1700 *g* for 10 min at room temperature, and the upper plasma layers were collected and stored at –80°C until analysis. Liver samples were then obtained and frozen in liquid N₂. All the samples were stored at –80°C until analysis. Furthermore, welfare-related assessments and interventions were carried out before, during and after the experiment by the Animal Research Committee.

Sample preparation for NMR analysis

Urine samples (540 μ l) were mixed with 60 μ l of PBS in 100% D₂O containing 500 mM NaP, 5 mM 3-(trimethylsilyl)propionic acid-d₄ sodium salt (TSP) and 0.04% (w/v) NaN₃. This mixture was centrifuged at 15 000 *g* for 5 min at 4°C, and 550 μ l supernatant was transferred to a 5 mm NMR tube (Shigemi). Plasma samples (540 μ l) were mixed with 60 μ l of PBS in 100% D₂O containing 500 mM NaP, 5 mM TSP, 10 mM formate and 0.04% (w/v) NaN₃ and centrifuged at 3000 *g* for 5 min at 4°C, and 550 μ l of supernatant was transferred to a 5 mm NMR tube. Liver samples were homogenised in 1.0 ml of extraction buffer (acetonitrile–MilliQ water = 1:1) per 100 mg of liver at 25 000 rpm for 30 s on ice. The homogenate was centrifuged at 5480 *g* for 10 min at 4°C, and 800 μ l of supernatant was collected and lyophilised. The lyophilised sample was suspended in PBS in 100% D₂O containing 200 mM NaP and 2 mM TSP. The suspension was centrifuged at 15 000 *g* for 5 min at 4°C, and the 550 μ l of supernatant was transferred to a 5 mm NMR tube. The pH of all supernatants was adjusted to 7.4. TSP and formate were used as internal standards. D₂O was used as an internal lock solvent.

NMR spectra acquisition

¹H NMR spectra were recorded using a Bruker 600 MHz AVANCE III spectrometer (Bruker) at a proton frequency of 600.13 MHz with the sample temperature controlled at 298 K. The ¹H NMR spectra of urine and liver samples were recorded using a water-suppressed standard one-dimensional NOESY1D pulse sequence, while those of the plasma samples were recorded using a water-suppressed standard one-dimensional CPMG pulse sequence. Each spectrum consisted of 32 768 data points with a spectral width of 12 parts per million (ppm). The acquisition time was 2.28 s, and the number of scans was 128. A water-suppressed pulse sequence was used to reduce the residual water signal at the water frequency with a recycle delay (D1 (Bruker notated)) of 2.72 s and a mixing time (D8 (Bruker notated)) of 0.10 s. A 90° pulse length was automatically calculated for each sample analysis.



Spectral data processing and multivariate data analysis

All raw spectra were manually corrected for phase and baseline distortions against TSP resonance at $\delta = 0.0$ ppm using Delta 5.0.4 (JEOL). The spectra were normalised to the peak area value of the internal standards TSP or formate using the NMR Suite 7.5 Processor (Chenomx). Differences in urine concentration between the samples were corrected by dividing each by their creatinine content. The normalised spectral data were exported to NMR Suite 7.5 Profiler (Chenomx). In the first round of processing, the 0.0–10.0 ppm chemical shift region was integrated into regions with a width of 0.04 ppm, while spectral regions related to residual water (4.68–5.08 ppm) and urea (5.50–6.00 ppm) were removed to eliminate their massive signals as it could affect other metabolite signals. In the second round of processing, metabolite assignment and quantification were determined by referencing the library from Chenomx NMR Suite and published literature^(20–22).

mRNA microarray experiments

As already described in our previous report⁽¹⁸⁾, total RNA was extracted from livers and purified. Complementary RNA was generated and labelled with cyanine 3 using a Low Input QuickAmp Labeling Kit (Agilent Technologies) and hybridised to a SurePrint G3 Rat GE microarray 8 × 60K (Agilent Technologies) according to the manufacturer's instructions. Fluorescence signals were detected using Agilent SureScan Microarray Scanner Extraction Software 12.0.3.1 (Agilent Technologies) according to the manufacturer's instructions. Raw data from the Feature Extraction Software were exported to GeneSpring GX version 14.9 (Agilent Technologies). To identify differentially expressed genes in the microarray hybridisation and reduce noise, each data set of fluorescence signals was normalised using a median shift algorithm (shifted to 75 percentile) with background correction to the median of all samples. Only genes with normalised signals detected on all microarrays were judged to be present. Furthermore, genes detected by the multiple probes were selected one to have the largest fold change. Subsequently, in order to find potential associations between the detected hepatic genes and identified metabolites, the Kyoto Encyclopedia of Genes and Genomes pathway database was used.

Gene ontology data analysis

For functional analysis of differentially expressed genes in mRNA microarray, up-regulated and down-regulated genes were subjected to gene set enrichment analysis with gene ontology (GO) terms using GeneSpring GX. GO term description and GO accession number were referenced to The Gene Ontology Resource, the web available bioinformatics database (<http://www.geneontology.org/>). Generally, Fisher's exact test and the χ^2 test were used to classify the GO term, and the false discovery rate was calculated to correct *P* value. *P* values of <0.05 were considered statistically significant.

Reverse transcription and quantitative PCR

To validate the gene fluctuations detected by mRNA microarray analysis, total RNA was reverse-transcribed using a QuantiTect

RT Kit (QIAGEN) and DNA templates were amplified using a CFX96 Real-Time PCR (Bio-Rad Laboratories) with TaqMan Fast Advanced Master Mix and TaqMan probes (Applied Biosystems) according to the manufacturer's instructions. The primers and probes are summarised in online Supplementary Table S1. Gene expression was normalised to β -actin and analysed using CFX Manager version 3.1 (Bio-Rad Laboratories).

Statistical analysis

The NMR spectral data matrix was exported into SIMCA-P 14.0 (Umetrics). Principal component analysis (PCA) was performed on the area values of binned NMR spectra as a non-targeting analysis. Score plots were obtained to visualise the clustering pattern of the samples along two principal components (PC1 and PC2), with each point denoting an individual sample spectrum. Unit variance was carried out on mean centred data. Supervised classification of orthogonal partial least squares discriminant analysis (OPLS-DA) was performed following PCA, to extract significant metabolite information. Loading plots indicated the metabolites responsible for group separation. Partial least square model quality was determined by the Q^2 value, which represents model predictability, and the model with Q^2 value of >0.5 generally indicates a good separation⁽²³⁾. Similarly, the cross-validated ANOVA (CV-ANOVA) *P* value is also used as an indicator of model quality, and the model with the *P* value of <0.05 indicates a statistically significant separation⁽²⁴⁾.

JMP software (version Pro 14.0.0, SAS Institute) was used for statistical analysis. Data for each dietary group are presented as the mean values and standard deviations. Two-way repeated-measures ANOVA was performed for urine metabolites data, and the statistical results (effects of time, diet and the interaction of time and diet) are presented in Fig. 3. Student's *t* tests with Welch's correction were used to compare the means of each group at each time point. *P* values of <0.05 were considered statistically significant.

Results

Characteristics of the experimental animals

As already described in our previous report⁽¹⁸⁾, while protein intake was significantly lower in the LP group than in the CT group (0.80 (SD 0.03) *v.* 1.87 (SD 0.08) g/d, *P* < 0.0001) and energy intake was significantly higher in the LP group than in the CT group (254 (SD 9) *v.* 217 (SD 9) kJ/d, *P* < 0.0001), no significant difference in body mass was observed between the CT and LP groups at week 4 (360 (SD 7.0) *v.* 353 (SD 7.8) g, *P* = 0.12). Despite that, the plantaris muscle mass tended to be lower in the LP group than in the CT group (1.68 (SD 0.08) *v.* 1.75 (SD 0.04) g/kg body mass, *P* = 0.06).

Urine metabolomic profiling

Representative ¹H NMR spectra of urine samples at week 4 are shown in online Supplementary Fig. S1. Low-molecular-weight compounds, such as amino acids (AA), AA-derived metabolites, tricarboxylic acid (TCA) cycle intermediates, taurine, choline and



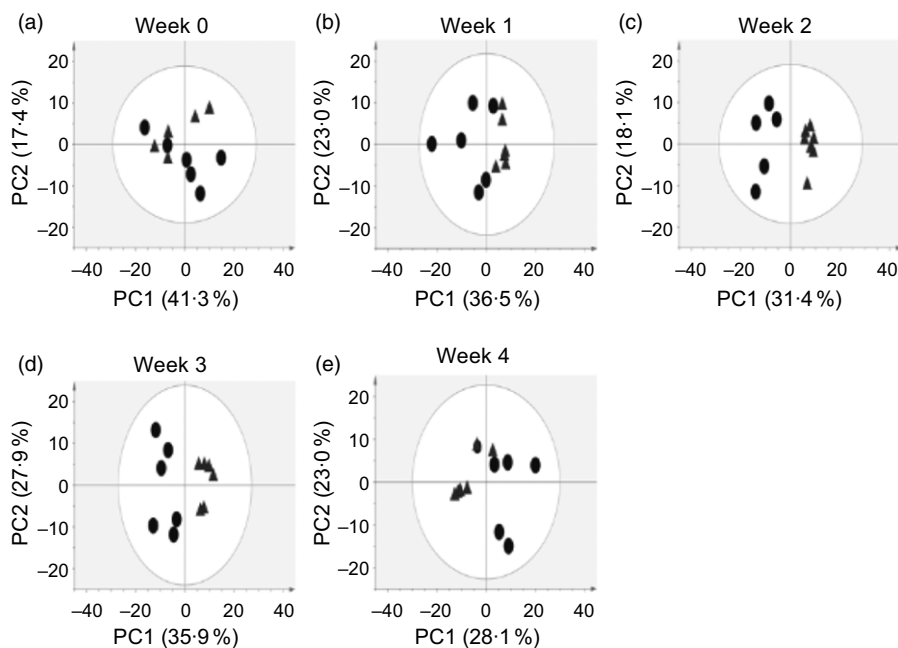


Fig. 1. Principal component (PC) analysis score plots derived from the NMR spectra of urine samples from rats fed control (CT) or low-protein (LP) diets at (a) week 0, (b) week 1, (c) week 2, (d) week 3 and (e) week 4. Each symbol represents the urine sample from an individual animal (n 6). ●, CT; ▲, LP.

trimethylamine N-oxide (TMAO), were identified, and their chemical shifts are shown in online Supplementary Table S2. Differences in the NMR spectra between the CT and LP groups were visualised using PCA at each time point (Fig. 1). The score plots derived from the urine NMR spectra of the two groups did not form separate clusters at week 0; however, the separation became apparent at weeks 1–4. Moreover, the score plots showed separation pattern by the components of PC1. In further multivariate analysis, pairwise comparisons of urine NMR spectra were performed using OPLS-DA at each time point to clarify the factors that contributed to separation by giving group information (Fig. 2). The Q^2 value, an indicator of the quality of OPLS-DA models, was maximal at week 3, and the CV-ANOVA P value, which assesses the validation of the OPLS-DA models, was also statistically significant at week 3. Urine metabolites with peaks containing correlation coefficients of 0.70 or more at specific time points from weeks 1–4 are illustrated in Fig. 2.

Relative concentrations of eighteen urine metabolites at weeks 0–4, responsible for differentiating these diet groups by OPLS-DA, were then examined (Fig. 3 and online Supplementary Table S3). Some essential AA (phenylalanine, threonine, tryptophan and valine), AA-derived metabolites (isobutyrate, cadaverine and putrescine), urea, taurine and N-methylnicotinamide were significantly lower in the LP group than in the CT group at specific time points from weeks 1–4. In contrast, TCA cycle intermediates (citrate, *cis*-aconitate, 2-oxoglutarate, succinate and fumarate), choline, tartrate and TMAO levels were significantly higher in the LP group than in the CT group at specific time points.

Plasma metabolomic profiling

Representative ^1H NMR spectra of plasma samples at week 4 are shown in online Supplementary Fig. S2. Assigned compounds

and their chemical shifts are shown in online Supplementary Table S4. The score plots derived from the plasma NMR spectra of rats fed CT or LP diets were not clearly separated in PCA (online Supplementary Fig. S3(a)). In further multivariate analysis, pairwise comparisons of plasma NMR spectra were performed using OPLS-DA (online Supplementary Fig. S3(b)). The Q^2 value was low (<0.5), and the CV-ANOVA P value was not statistically significant. Plasma metabolites, with peaks containing correlation coefficients of 0.70 or more, were glutamine, serine, valine and urea. The relative concentration of plasma metabolites that were detected in rats fed a CT or LP diet for 4 weeks is shown in online Supplementary Table S5. Plasma betaine, fumarate, glutamine, glycine, N,N-dimethylglycine and serine levels were significantly higher in the LP group than in the CT group at week 4, whereas isoleucine, leucine, phenylalanine, threonine, urea and valine levels were significantly lower in the LP group than in the CT group at week 4. Among them, five metabolites (phenylalanine, threonine, valine, urea and fumarate) were also identified in the urine and these levels in plasma are shown in Fig. 4.

Liver metabolomic profiling

Representative ^1H NMR spectra of liver samples at week 4 are shown in online Supplementary Fig. S4. Assigned compounds and their chemical shifts are shown in online Supplementary Table S6. Among metabolites identified in both urine and liver, some were not identified in plasma. This may be due to these metabolites binding to proteins in the blood, which would affect the pulse sequence to eliminate signals derived from protein in the NMR analysis of plasma as discussed in a previous report⁽²⁵⁾. The score plots derived from the liver NMR spectra of the two groups were clearly separated by the components of PC2 in

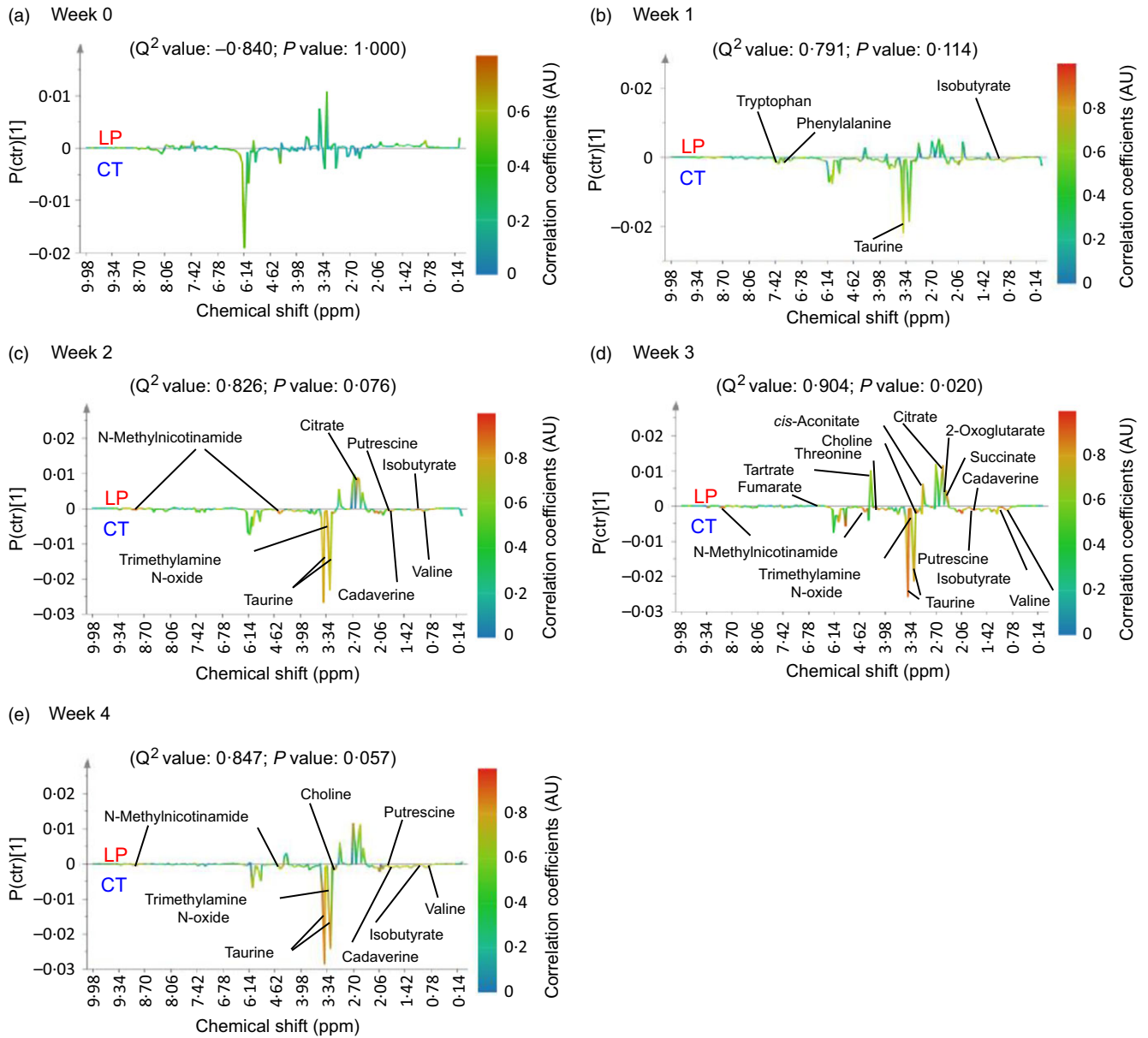


Fig. 2. Multivariate analysis of binned NMR spectra of urine samples from rats fed control (CT) or low-protein (LP) diets at weeks 0–4. Coefficient loading plots obtained from rats fed CT or LP diets at (a) week 0, (b) week 1, (c) week 2, (d) week 3 and (e) week 4. Peaks pointing upwards indicate metabolites whose levels were higher in the LP group than in the CT group, whereas peaks pointing downwards indicate metabolites whose levels were higher in the CT group than in the LP group. Heat colours indicate the contribution degree of metabolites that separate the CT and LP groups. Among the assigned metabolites, those with NMR spectra with correlation coefficients of 0.70 or more are annotated in the figure. Partial least square model quality was determined by Q^2 value, which represent model predictability, and the P value of cross-validated ANOVA. Each value is shown in the figure. ppm, Parts per million.

the PCA (online Supplementary Fig. S5(a)). In further multivariate analysis, pairwise comparisons of liver NMR spectra were performed using OPLS-DA (online Supplementary Fig. S5(b)). The Q^2 value was high (≥ 0.5), and the CV-ANOVA P value was statistically significant. Liver metabolites, with peaks containing correlation coefficients of 0.70 or more, were inosine, uridine, NAD^+ , taurine and hypoxanthine. The relative concentration of liver metabolites that were detected in rats fed CT or LP diets for 4 weeks is shown in online Supplementary Table S7. Liver β -alanine, choline, dimethylamine, hypoxanthine, inosine monophosphate and TMAO levels were significantly higher in the LP group than in the CT group at week 4, whereas inosine,

nicotinamide adenine, NAD^+ , α -phosphocholine, taurine and uridine levels were significantly lower in the LP group than in the CT group at week 4. Among them, three metabolites (taurine, TMAO and choline) were also identified in the urine and these levels in the liver are shown in Fig. 5.

mRNA microarray for hepatic transcriptomic profiling

When GO analysis following hepatic mRNA microarray was performed to investigate how metabolite fluctuations occurred, the metabolic process pathway was determined to be most significantly altered among the biological processes. The metabolic

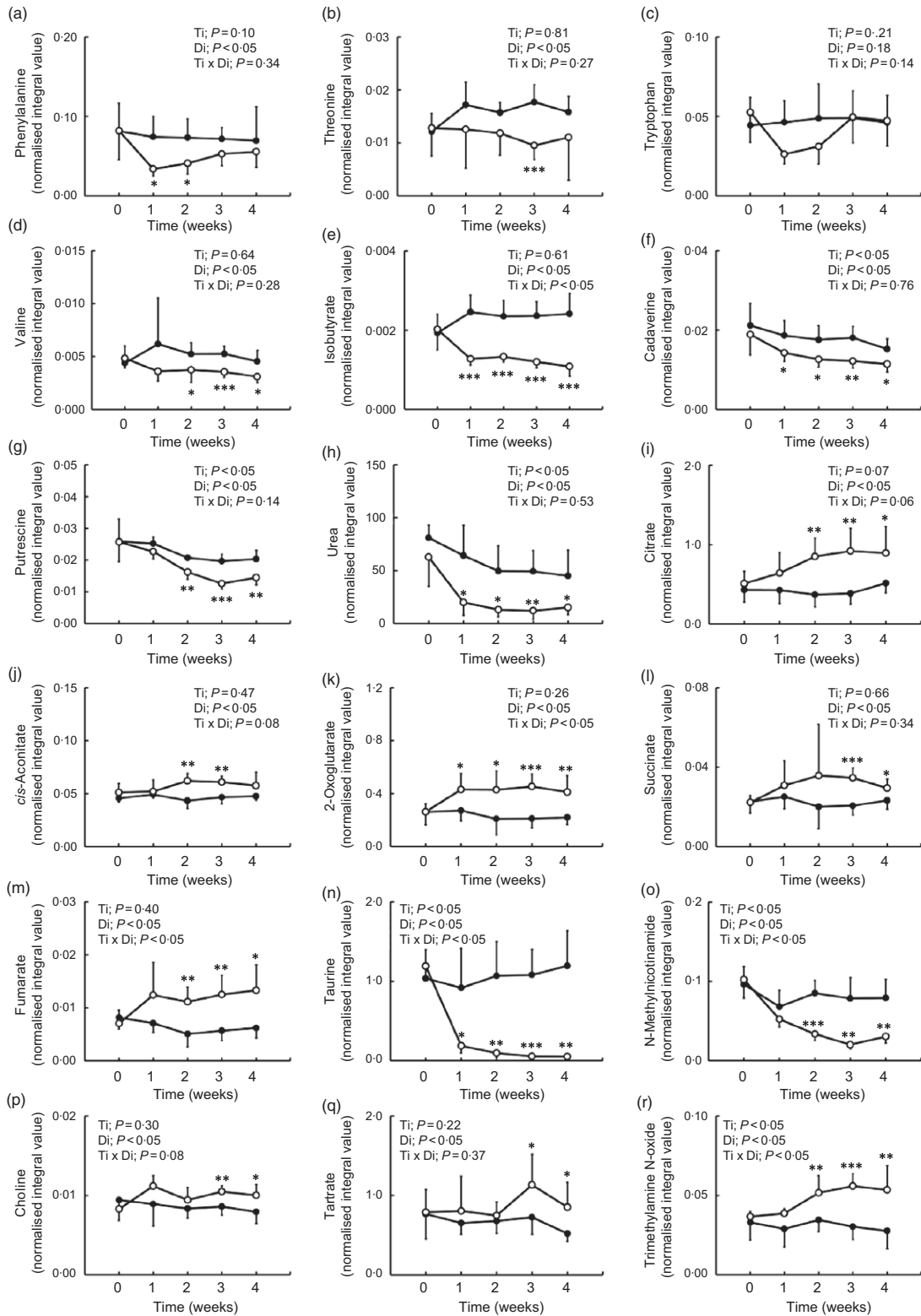


Fig. 3. Relative concentrations of urinary metabolites responsible for differentiating the control (CT) and low-protein (LP) diet groups by orthogonal partial least squares discriminant analysis in urine samples at weeks 0–4. The levels of (a) phenylalanine, (b) threonine, (c) tryptophan, (d) valine, (e) isobutyrate, (f) cadaverine, (g) putrescine, (h) urea, (i) citrate, (j) *cis*-aconitate, (k) 2-oxoglutarate, (l) succinate, (m) fumarate, (n) taurine, (o) N-methylnicotinamide, (p) choline, (q) tartrate and (r) trimethylamine N-oxide are shown. The relative concentration of each metabolite is its integral value normalised to 3-(trimethylsilyl)propionic acid-d4 sodium salt (TSP), which was added to the NMR buffer as an internal standard. Data are mean values and standard deviations ($n=6$) and were analysed by Student's *t* tests with Welch's correction. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. —●—, CT; —○—, LP. Ti, time; Di, diet.



Metabolites change in protein undernutrition

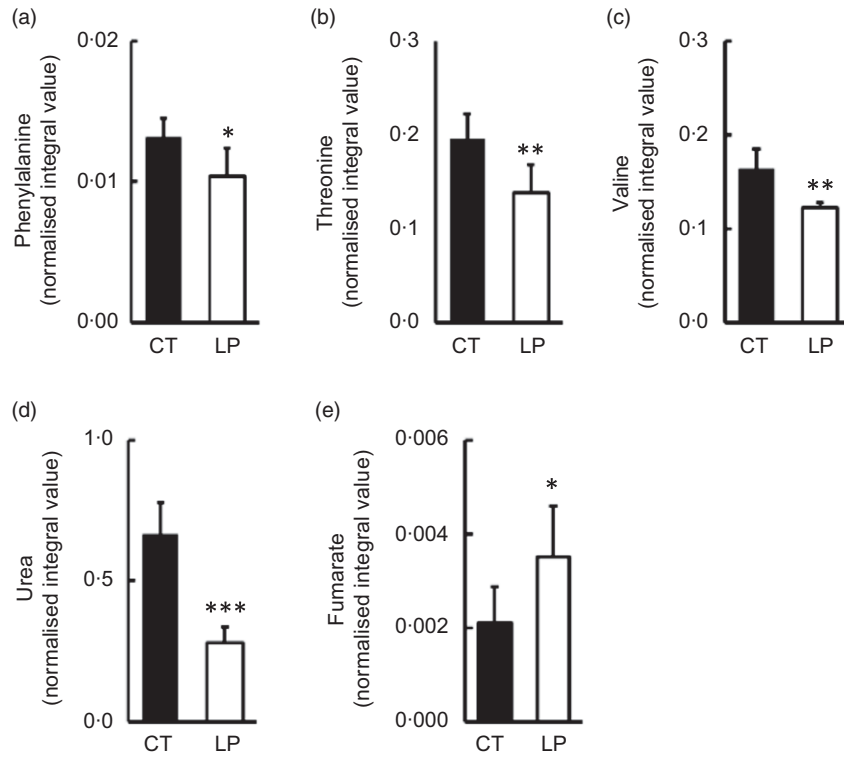


Fig. 4. Relative concentrations of metabolites in plasma samples of rats fed control (CT) or low-protein (LP) diets for 4 weeks. The levels of (a) phenylalanine, (b) threonine, (d) valine, (d) urea and (e) fumarate are shown. The relative concentration of each metabolite is its integral value normalised to formic acid, which was added to the NMR buffer as an internal standard. Data are mean values and standard deviations (n 6). P values for the effects of time (Ti), diet (Di) and the interaction of time and diet (Ti×Di) were calculated by a two-way repeated-measures ANOVA and were shown in each subfigure. Student's t tests with Welch's correction were used to compare the means of diet group at each time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ■, CT; □, LP.

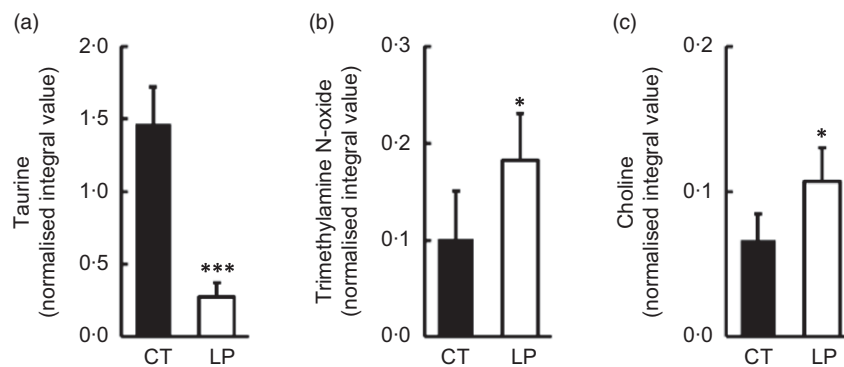


Fig. 5. Relative concentrations of metabolites in liver samples of rats fed control (CT) or low-protein (LP) diets for 4 weeks. The levels of (a) taurine, (b) trimethylamine N-oxide and (c) choline are shown. The relative concentration of each metabolite is its integral value normalised to 3-(trimethylsilyl)propionic acid-d4 sodium salt (TSP), which was added to the NMR buffer as an internal standard. Data are mean values and standard deviations (n 6) and were analysed by Student's t tests with Welch's correction. * $P < 0.05$, *** $P < 0.001$. ■, CT; □, LP.

process pathway encompasses several sub-pathways, including cellular metabolic process, the organic substance metabolic process and the biosynthetic process (Table 1).

mRNA microarray analysis revealed that a total of 19 598 gene expressions were confirmed in the livers, of which 3056 were significantly differentially expressed between the CT and LP groups. Among them, 352 genes were significantly over-expressed in the LP group than in the CT group by more than 1.5-fold, whereas 362 genes were significantly under-expressed

in the LP group than in the CT group by more than 1.5-fold (online Supplementary Table S8). Gene expressions associated with eight metabolites that were identified in both urine and plasma/liver were further analysed by quantitative PCR to validate the microarray findings (Fig. 6). The gene expression levels of argininosuccinate lyase (*Asl*), argininosuccinate synthase 1 (*Ass1*) and cysteine dioxygenase type 1 (*Cdo1*) were significantly lower in the LP group than in the CT group. In contrast, the gene expression levels of glucose-6-phosphate isomerase,

Table 1. Gene ontology (GO) terms for functional analysis of differentially expressed genes*

GO term description	Number of genes	P
GO:0008150 Biological process		
GO:0008152 Metabolic process	444	<0.05
GO:0044237 Cellular metabolic process	409	<0.05
GO:0071704 Organic substance metabolic process	423	<0.05
GO:0009058 Biosynthetic process	226	<0.05
GO:0044281 Small molecule metabolic process	141	<0.05
GO:0044238 Primary metabolic process	399	<0.05
GO:0071840 Cellular component organisation or biogenesis	223	0.16
GO:0009987 Cellular process	562	1.00
GO:0065007 Biological regulation	393	1.00

* GO term description and GO accession number were referenced to The Gene Ontology Resource, the web-available bioinformatics database (<http://www.geneontology.org>). P values of <0.05 were considered statistically significant (Fisher's exact test).

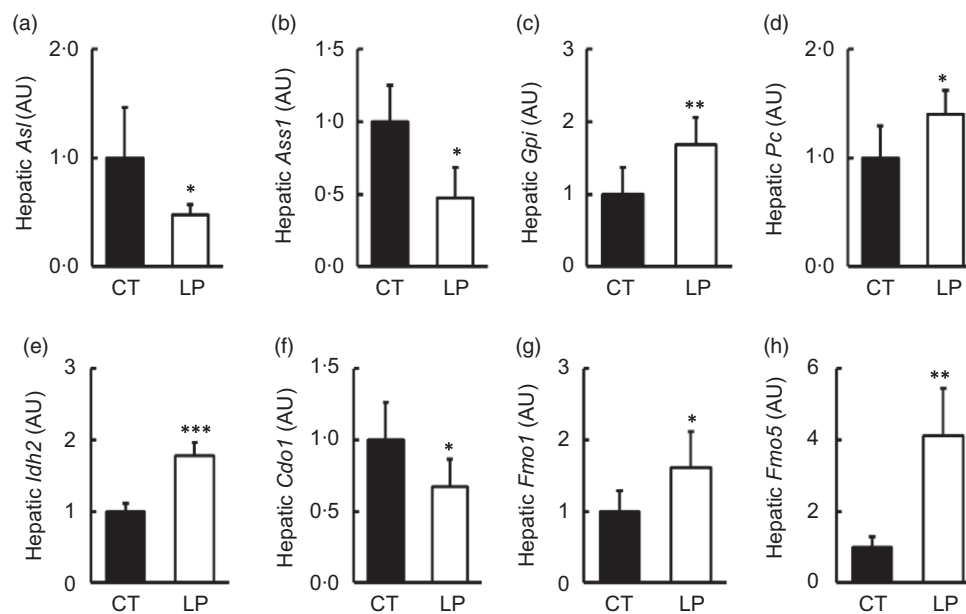


Fig. 6. Hepatic gene expression levels in rats fed control (CT) or low-protein (LP) diets for 4 weeks. Relative expression levels of (a) argininosuccinate lyase (*Asl*), (b) argininosuccinate synthase 1 (*Ass1*), (c) glucose-6-phosphate isomerase (*Gpi*), (d) pyruvate carboxylase (*Pc*), (e) isocitrate dehydrogenase 2 (*Idh2*), (f) cysteine dioxygenase type 1 (*Cdo1*), (g) flavin containing mono-oxygenase 1 (*Fmo1*) and (h) *Fmo5* were examined at week 4. Among genes differentially expressed between the CT and LP diet groups by mRNA microarray, only those related to fluctuating metabolites were examined. Values are normalised to the CT group. Data are mean values and standard deviations ($n = 6$) and were analysed by Student's *t* tests with Welch's correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ■, CT; □, LP.

pyruvate carboxylase, isocitrate dehydrogenase 2, flavin-containing mono-oxygenase 1 (*Fmo1*) and *Fmo5* were significantly higher in the LP group than in the CT group.

Discussion

We performed metabolomic and transcriptomic analyses in adult rats fed a 14% casein diet (CT diet) or 5% casein diet (LP diet) for 4 weeks. According to our previous report using the same experimental animals as this study⁽¹⁸⁾, no significant differences in body mass were observed between the two groups, whereas plantaris muscle mass tended to be lower in the LP group than in the CT group ($P = 0.06$), implicating the manifestation of potential protein undernutrition in the LP group. Furthermore, GO analysis indicated that protein undernutrition perturbed metabolic processes such as the cellular metabolic process, the

organic substance metabolic process and the biosynthetic process, which is likely, in turn, to lead to fluctuations in metabolite levels. Thus, the novelty of this study was to identify metabolites as biomarker candidates responsive to protein undernutrition using metabolomics and transcriptomic approaches.

Metabolites identified in both urine and plasma were phenylalanine, threonine, valine, urea and fumarate. In urine and plasma, the levels of AA and urea were significantly lower in the LP group than in the CT group, respectively. These observations are presumed to be caused by reduced AA/protein intake. Furthermore, lowered urinary phenylalanine excretions in rats fed the LP diet were observed at weeks 1 and 2, whereas lowered threonine excretion was observed at week 3. Hence, different durations of protein undernutrition may impact the pattern of AA/protein metabolism. In line with AA excretion, hepatic expression levels of *Asl* and *Ass1*, which encode enzymes in the urea cycle, were significantly lower in the LP group than

in the CT group. This is probably due to the decreased metabolism of AA-derived nitrogen, which would stagnate the urea cycle. Similarly, decreased protein intake is reported to reduce the hepatic expression of genes which encode urea cycle-related enzymes⁽²⁶⁾. Protein insufficiency may facilitate nitrogen conservation in tissues and decrease hepatic *Asl* and *Ass1* expressions in the LP group. In contrast, fumarate levels in urine and plasma were significantly higher in the LP group than in the CT group, respectively. The observed increase in fumarate, a TCA intermediate, may be due to higher carbohydrate contents in the LP diet. Hepatic gene expression levels of glycolytic enzymes glucose-6-phosphate isomerase, pyruvate carboxylase and the TCA cycle enzyme isocitrate dehydrogenase 2 were also significantly higher in the LP group than in the CT group. These suggest that glucose metabolism was activated by increased carbohydrate intake accompanied by high carbohydrate contents in the LP diet. However, it should be noted that TCA cycle acceleration following high carbohydrate intake has been linked to the progression of various diseases, such as cancer and atherosclerosis, through the overproduction of reactive oxygen species in the mitochondria⁽²⁷⁾. Furthermore, fumarate accumulation can lead to oxidative stress by modifying glutathione metabolism⁽²⁸⁾ and induce endoplasmic reticulum stress in a diabetic model⁽²⁹⁾. Thus, the increase in the TCA cycle activity and its intermediate fumarate associated with LP diet ingestion may increase the risk of developing specific diseases.

The OPLS-DA showed that the Q^2 value of liver samples was higher than that of plasma samples, and the CV-ANOVA P value of liver samples was statistically significant, suggesting that the liver reflected protein undernutrition more substantially than plasma. Thus, metabolites such as taurine, TMAO and choline, which fluctuated not only in urine but also in the liver, may be sensitive to protein undernutrition. Notably, urinary taurine levels were significantly lower in the LP group than in the CT group at week 1 and remained constant thereafter. The liver taurine level was also significantly lower in the LP group than in the CT group at week 4. The activity of CDO1, the rate-limiting enzyme in the biosynthesis of taurine from cysteine⁽³⁰⁾, is considerably higher in the liver than other tissues⁽³¹⁾. Thus, decreased urinary and hepatic taurine levels could have been caused by the stagnation of cysteine metabolism associated with reduced hepatic expression of *Cdo1*. Taurine contributes to numerous biological functions through its anti-oxidative and anti-inflammatory properties⁽³²⁾. It is reported that taurine deficiency induces skeletal muscle loss⁽³³⁾, while taurine intake improves skeletal muscle function by preventing muscle degeneration^(34,35). Muscle degeneration is related to oxidative stress in the muscle and promotes muscle cell apoptosis. It is reported that taurine can protect against oxidative stress as well as reducing the abundance of apoptotic signal proteins^(36,37). In our previous study⁽¹⁸⁾ examining the same experimental animals in this study, the plantaris muscle mass tended to be lower in the LP group than in the CT group ($P=0.06$). Collectively, this might be mediated in part by decreased taurine levels in the body. Although gene expression analyses involved in muscle synthesis and degradation in muscle tissues are lacking in this study, it would be necessary to investigate the molecular mechanism to clarify the relationship between taurine concentration and

muscle mass. Taurine deficiency is also reported to affect energy metabolism in adipose tissue⁽³⁸⁾. Taurine protects against dyslipidaemia by inhibiting reactive oxygen species generation^(39,40), and taurine supplementation can normalise serum HDL concentrations in a high cholesterol diet-fed rats⁽⁴¹⁾. It has also been reported that protein intake and serum HDL-cholesterol levels are positively correlated⁽⁴²⁾. Furthermore, in our previous study using the same experimental animals as in the present study⁽¹⁸⁾, the LP group exhibited significantly lower HDL-cholesterol compared with the CT group. Considering these facts, taurine level may be involved in dyslipidaemia associated with protein undernutrition. In contrast to the decreased taurine level in urine and liver of the LP group, urinary TMAO levels were significantly higher in the LP group than in the CT group at week 2 and it remained constant thereafter. The liver TMAO level was also significantly higher in the LP group than in the CT group at week 4. In our metabolomic analysis, choline in urine and liver were also higher in the LP group than in the CT group. Choline is converted to TMA by intestinal bacteria, and TMAO is synthesised from TMA by the FMO family in the liver⁽⁴³⁾. Hepatic gene expressions of *Fmo1* and *Fmo5* were also significantly higher in the LP group than in the CT group, respectively. Therefore, it is speculated that the increase in TMAO level was associated with an increase in both its substrate choline and the gene expression levels of *Fmo1* and 5. The urinary tartrate level was also significantly higher in the LP group than in the CT group. Since choline was provided in both CT and LP diets as choline bitartrate, the increased urinary tartrate excretion observed in the LP-fed rats may indicate the use of choline as a nitrogen source in protein undernutrition, as observed previously⁽⁴⁴⁾. Besides, as protein deficiency has been suggested to alter the gut microbiota^(44,45), increased TMAO levels may also be indirectly caused by fluctuations in intestinal microbiota. Clinical trials have demonstrated that elevated TMAO level increases the risk of CVD^(43,46), while consumption of a high TMAO-containing diet reduces serum HDL levels in mice⁽⁴⁷⁾. A recent review suggests that TMAO-induced atherosclerosis is associated with decreased HDL-cholesterol, which increases the risk of CVD⁽⁴⁸⁾. Furthermore, as mentioned above, our previous study using the same animals reported that protein undernutrition reduced HDL-cholesterol⁽¹⁸⁾. Thus, the increase in TMAO levels in the LP group may also be associated with decreased circulating HDL-cholesterol levels. Collectively, the increase in choline utilisation as a nitrogen source induced by protein undernutrition may increase the risk of dyslipidaemia and cardiovascular disorder via elevating TMAO production. It should also be noted that the plasma TMAO level is affected by the intake of fish containing TMAO and *de novo* synthesis from carnitine contained in red meat^(49,50). Therefore, TMAO intake derived from diets should be taken into account for comprehending the TMAO level fluctuation in the body.

In conclusion, the fluctuation of several metabolites identified in urine, plasma and liver samples was caused by the LP diet ingestion in adult rats. Among the identified metabolites, taurine and TMAO, the final metabolites that are not further metabolised, fluctuated not only in urine but also in the liver. Notably, changes in taurine and TMAO levels were detectable in the urine by week 2 after LP diet ingestion, which may reflect protein



undernutrition at an early stage. Hepatic gene expressions of taurine and TMAO biosynthesis enzymes also corresponded to these fluctuations. Thus, fluctuations in urinary taurine and TMAO are considered to have occurred as a result of substantial responses to protein undernutrition in the liver. Furthermore, taurine and TMAO might also be potential mediators of the development of skeletal muscle disorders and lipid abnormalities, which could be associated with LP nutritional status as discussed above. It follows from these results that taurine and TMAO may serve as potential biomarker candidates of protein undernutrition and targets for prevention of secondary diseases associated with protein undernutrition. To further elucidate in-depth the molecular mechanisms involved in taurine and TMAO in the context of protein nutritional status, it may be necessary to determine whether fluctuations in taurine and TMAO levels dissolve as the protein nutritional status improves. In addition, clinical trials are warranted to investigate their usefulness as protein nutrition biomarkers.

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Supplementary material

For supplementary materials referred to in this article, please visit <https://doi.org/10.1017/S0007114520003281>

Metabolomics and transcriptomics raw data are available from MetaboLights (Study Identifier: MTBLS1452, <https://www.ebi.ac.uk/metabolights/MTBLS1452>) and Gene Expression Omnibus (Study Identifier: GSE144207, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144207>).

References

- Henchion M, Hayes M, Mullen AM, *et al.* (2017) Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods* **6**, E53.
- Deutz NEP, Bauer JM, Barazzoni R, *et al.* (2014) Protein intake and exercise for optimal muscle function with aging: recommendations from the ESPEN Expert Group. *Clin Nutr* **33**, 929–36.
- Landi F, Calvani R, Tosato M, *et al.* (2016) Protein intake and muscle health in old age: from biological plausibility to clinical evidence. *Nutrients* **8**, 295.
- Yamada R, Odamaki S, Araki M, *et al.* (2019) Dietary protein restriction increases hepatic leptin receptor mRNA and plasma soluble leptin receptor in male rodents. *PLOS ONE* **14**, e0219603.
- Nishi H, Yamanaka D, Kamei H, *et al.* (2018) Importance of serum amino acid profile for induction of hepatic steatosis under protein malnutrition. *Sci Rep* **8**, 5461.
- Taguchi Y, Toyoshima Y, Tokita R, *et al.* (2017) Triglyceride synthesis in hepatocytes isolated from rats fed a low-protein diet is enhanced independently of upregulation of insulin signaling. *Biochem Biophys Res Commun* **490**, 800–805.
- Toyoshima Y, Tokita R, Taguchi Y, *et al.* (2014) Tissue-specific effects of protein malnutrition on insulin signaling pathway and lipid accumulation in growing rats. *Endocr J* **61**, 499–512.
- Zhang Z, Pereira SL, Luo M, *et al.* (2017) Evaluation of blood biomarkers associated with risk of malnutrition in older adults: a systematic review and meta-analysis. *Nutrients* **9**, E829.
- Omran ML & Morley JE (2000) Assessment of protein energy malnutrition in older persons, Part II: laboratory evaluation. *Nutrition* **16**, 131–140.
- Gowda S, Desai PB, Kulkarni SS, *et al.* (2010) Markers of renal function tests. *N Am J Med Sci* **2**, 170–173.
- Bingham SA (2003) Urine nitrogen as a biomarker for the validation of dietary protein intake. *J Nutr* **3**, 921S–924S.
- Marliss EB, Wei CN & Dietrich LL (1979) The short-term effects of protein intake on 3-methylhistidine excretion. *Am J Clin Nutr* **32**, 1617–1621.
- Stella C, Beckwith-Hall B, Cloarec O, *et al.* (2006) Susceptibility of human metabolic phenotypes to dietary modulation. *J Proteome Res* **5**, 2780–2788.
- Abate-Shen C & Shen MM (2009) Diagnostics: the prostate-cancer metabolome. *Nature* **457**, 799–800.
- Brindle JT, Antti H, Holmes E, *et al.* (2002) Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ¹H-NMR-based metabolomics. *Nat Med* **8**, 1439–1444.
- Jones DP, Park Y & Ziegler TR (2012) Nutritional metabolomics: progress in addressing complexity in diet and health. *Annu Rev Nutr* **32**, 183–202.
- Moco S, Bino RJ, De Vos RCH, *et al.* (2007) Metabolomics technologies and metabolite identification. *Trends Anal Chem* **26**, 855–866.
- Wada Y, Izumi H, Shimizu T, *et al.* (2020) A more oxidized plasma albumin redox state and lower plasma HDL particle number reflect low-protein diet ingestion in adult rats. *J Nutr* **150**, 256–266.
- Wada Y, Komatsu Y, Izumi H, *et al.* (2018) Increased ratio of non-mercaptalbumin-1 among total plasma albumin demonstrates potential protein undernutrition in adult rats. *Front Nutr* **5**, 64.
- Xiong Z, Lang L, Gao X, *et al.* (2019) An integrative urinary metabolomic study of the therapeutic effect of Guizhi Fuling capsule on primary dysmenorrheal rats based ¹H NMR and UPLC-MS. *J Pharm Biomed Anal* **164**, 750–758.
- Wang LF, Hu XJ, Peng RY, *et al.* (2012) Application of ¹H-NMR-based metabolomics for detecting injury induced by long-term microwave exposure in Wistar rats' urine. *Anal Bioanal Chem* **404**, 69–78.
- Ebbels TM, Holmes E, Lindon JC, *et al.* (2004) Evaluation of metabolic variation in normal rat strains from a statistical analysis of ¹H NMR spectra of urine. *J Pharm Biomed Anal* **36**, 823–833.
- MKS Umetrics (2012) *User Guide to SIMCA Version 13*. <http://chemsrv0.pph.univie.ac.at/scripten/EDV/Software/Simca13/User%20Guide%20to%20SIMCA%2013.pdf> (accessed September 2020).



24. Triba MN, Le Moyec L, Amathieu R, *et al.* (2015) PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. *Mol Biosyst* **11**, 13–19.
25. de Graaf RA & Beharn KL (2003) Quantitative ¹H NMR spectroscopy of blood plasma metabolites. *Anal Chem* **75**, 2100–2104.
26. Young VR & Marchini JS (1990) Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *Am J Clin Nutr* **51**, 270–289.
27. Matsuda M & Shimomura I (2013) Increased oxidative stress in obesity: implications for metabolic syndrome, diabetes, hypertension, dyslipidemia, atherosclerosis, and cancer. *Obes Res Clin Pract* **7**, e330–e341.
28. Zheng L, Cardaci S, Jerby L, *et al.* (2015) Fumarate induces redox-dependent senescence by modifying glutathione metabolism. *Nat Commun* **6**, 6001.
29. You YH, Quach T, Saito R, *et al.* (2016) Metabolomics reveals a key role for fumarate in mediating the effects of NADPH oxidase 4 in diabetic kidney disease. *J Am Soc Nephrol* **27**, 466–481.
30. Hayes KC & Sturman JA (1981) Taurine in metabolism. *Annu Rev Nutr* **1**, 401–425.
31. Stipanuk MH (2004) Role of the liver in regulation of body cysteine and taurine levels: a brief review. *Neurochem Res* **29**, 105–110.
32. Marcinkiewicz J & Kontny E (2014) Taurine and inflammatory diseases. *Amino Acids* **46**, 7–20.
33. De Luca A, Pierno S & Camerino DC (2015) Taurine: the appeal of a safe amino acid for skeletal muscle disorders. *J Transl Med* **13**, 243.
34. Seidel U, Huebbe P & Rimbach G (2019) Taurine: a regulator of cellular redox homeostasis and skeletal muscle function. *Mol Nutr Food Res* **63**, e1800569.
35. Terrill JR, Pinniger GJ, Graves JA, *et al.* (2016) Increasing taurine intake and taurine synthesis improves skeletal muscle function in the mdx mouse model for Duchenne muscular dystrophy. *J Physiol* **594**, 3095–3110.
36. Khalil RM, Abdo WS, Saad A, *et al.* (2018) Muscle proteolytic system modulation through the effect of taurine on mice bearing muscular atrophy. *Mol Cell Biochem* **444**, 161–168.
37. Takatani T, Takahashi K, Uozumi Y, *et al.* (2004) Taurine inhibits apoptosis by preventing formation of the Apaf-1/caspase-9 apoptosome. *Am J Physiol Cell Physiol* **287**, C949–C953.
38. Murakami S (2015) Role of taurine in the pathogenesis of obesity. *Mol Nutr Food Res* **59**, 1353–1363.
39. Wen C, Li F, Zhang L, *et al.* (2019) Taurine is involved in energy metabolism in muscles, adipose tissue, and the liver. *Mol Nutr Food Res* **63**, e1800536.
40. Scicchitano BM & Sica G (2018) The beneficial effects of taurine to counteract sarcopenia. *Curr Protein Pept Sci* **19**, 673–680.
41. Yokogoshi H, Mochizuki H, Nanami K, *et al.* (1999) Dietary taurine enhances cholesterol degradation and reduces serum and liver cholesterol concentrations in rats fed a high-cholesterol diet. *J Nutr* **129**, 1705–1712.
42. Pasiakos SM, Lieberman HR & Fulgoni VL (2015) Higher-protein diets are associated with higher HDL cholesterol and lower BMI and waist circumference in US adults. *J Nutr* **145**, 605–614.
43. Tang WH, Wang Z, Levison BS, *et al.* (2013) Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med* **368**, 1575–1584.
44. Mayneris-Perxachs J, Bolick DT, Leng J, *et al.* (2016) Protein- and zinc-deficient diets modulate the murine microbiome and metabolic phenotype. *Am J Clin Nutr* **104**, 1253–1262.
45. Ma N, Tian Y, Wu Y, *et al.* (2017) Contributions of the interaction between dietary protein and gut microbiota to intestinal health. *Curr Protein Pept Sci* **18**, 795–808.
46. Randrianarisoa E, Lehn-Stefan A, Wang X, *et al.* (2016) Relationship of serum trimethylamine N-oxide (TMAO) levels with early atherosclerosis in humans. *Sci Rep* **6**, 26745.
47. Hu Y, Zhao Y, Yuan L, *et al.* (2015) Protective effects of tartary buckwheat flavonoids on high TMAO diet-induced vascular dysfunction and liver injury in mice. *Food Funct* **6**, 3359–3372.
48. Canyelles M, Tondo M, Cedó L, *et al.* (2018) Trimethylamine N-oxide: a link among diet, gut microbiota, gene regulation of liver and intestine cholesterol homeostasis and HDL function. *Int J Mol Sci* **19**, E3228.
49. Cho CE, Taesuan S, Malysheva OV, *et al.* (2017) Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: a randomized controlled trial. *Mol Nutr Food Res* **61**, 1600324.
50. Koeth RA, Wang Z, Levison BS, *et al.* (2013) Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* **19**, 576–585.