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ORIGINAL ARTICLE

Analysis of urinary sphingolipids using liquid chromatography-tandem mass spectrometry in diabetic nephropathy

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Ceramide, Tandem mass spectrometry, Urine

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ABSTRACT

Aims/Introduction: Sphingolipids, such as ceramides and sphingosine, are involved in the pathogenesis of diabetes; however, the modulation of urinary sphingolipids in diabetic nephropathy has not been fully elucidated. Therefore, we aimed to develop a simultaneous measurement system for urinary sphingolipids using liquid chromatography-tandem mass spectrometry and to elucidate the modulation of urinary sphingolipids in diabetic nephropathy.

Materials and Methods: We established a simultaneous measurement system for the urinary sphingosine, dihydrosphingosine, and six ceramide species (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0), and we examined the urinary sphingolipids in 64 type 2 diabetes patients and 15 control participants.

Results: The established measurement system for the urinary sphingolipids showed good precision for Cer d18:1/16:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0. We observed that the urinary levels of Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0 were elevated in patients with stage 3 of diabetic nephropathy, and were correlated with urinary biomarkers, such as albumin and *N*-acetyl-β-D-glucosaminidase, and sediment score.

Conclusions: Our method is useful for the measurement of ceramide in urine specimens, and urinary ceramides might be associated with the pathological condition of diabetic nephropathy, such as renal tubular injury.

INTRODUCTION

Sphingolipids are a class of lipids that possess a sphingoid base as a backbone. Among the sphingolipids, both ceramides and sphingosine 1-phosphate have been shown to possess important physiological roles. Ceramides have a sphingoid backbone to which a single fatty acid of various possible lengths and degrees of saturation is attached[1–3]. The class of the fatty acid chain determines the species of ceramide. Ceramides have been reported to be produced by the de novo pathway in the endoplasmic reticulum or by the salvage pathway in lysosomes, and they can be converted into various sphingolipids, including sphingosine (Sph), which is a substrate for sphingosine 1-phosphate and is another bioactive sphingolipid[4]. Reportedly, ceramides and other sphingolipids possess important roles in various pathophysiological conditions, including stress responses, apoptosis, inflammation and proliferation[4–8]. Therefore, they are thought to be involved in the pathogenesis of various diseases, including diabetes mellitus[9–11], neurodegenerative disorders[12], multiple sclerosis[13] and coronary artery disease[14].

Many studies have reported the potential roles of ceramides and Sphs in the pathogenesis of diabetes, such as their involvement in insulin resistance[9–11], pancreatic β-cell function, dysfunction and apoptosis[15–17]. In addition to these associations between ceramides or Sphs and the pathogenesis of disturbances in glucose homeostasis, several studies have reported the

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role of ceramide in the pathogenesis of kidney diseases; for example, ceramide has been shown to be involved in the apoptosis of renal mesangial cells[18] and renal tubular epithelial cells (RTEs)[19,20], which are observed in patients with diabetic nephropathy. However, although several studies have reported that plasma ceramide levels, including C18:0, C20:0 and C24:1, were elevated in diabetic nephropathy[9–11], only a limited number of reports have investigated the association between urinary ceramide and/or Sph levels and diabetic nephropathy using either a diabetic animal model or a very small number of children with type 1 diabetes[21,22].

To investigate the association between diabetic nephropathy and ceramides or Sphs, some analytical methodologies have been developed, including immunochemical methods[23,24] and gas chromatography[25]. In addition to these assays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) enables the rapid and highly sensitive lipid analysis monitoring of molecular species[26–28].

Considering these backgrounds, in the present study, we aimed to develop a highly sensitive and high-throughput measurement system using LC-MS/MS to quantify urinary sphingolipids. With this method, we measured the concentrations of ceramides and Sphs in urine collected from diabetes patients, and compared their concentrations with the clinical phenotypes of diabetic nephropathy, such as the presence of these particles in urine sediment.

METHODS

Patients and urine sampling

We enrolled 64 patients with type 2 diabetes mellitus who regularly attend the University of Tokyo Hospital, Tokyo, Japan. The patients were clinically diagnosed as having diabetes mellitus, and the presence and stages of nephropathy were assessed. We defined the stages of diabetic nephropathy according to the Classification of Diabetic Nephropathy 2014, published by the Research Group of Diabetic Nephropathy in Japan[29,30]. As the normal control group, we enrolled 15 individuals who did not have diabetes, and whose urinary albumin levels were <30 mg/gCr and estimated glomerular levels were >60 mL/min per 1.73 m^2. Urine specimens were collected after a clinical examination, and the supernatant was stored at −80°C until measurement. We obtained written informed consent from all the participants, and this study was approved by the ethics committee of the University of Tokyo (10266).

Analysis of ceramides using LC-MS/MS

For the LC-MS/MS analysis, 10 μL of urine was mixed with 190 μL of methanol (Wako Pure Chemical Industries, Osaka, Japan) acidified with 0.1% formic acid including an internal standard. C17:1 Sph (Avanti Polar Lipids, Alabaster, AL, USA), C17:1 dihydrosphingosine (dhSph; Avanti Polar Lipids) and C17 ceramide (d18:1/17:0; Avanti Polar Lipids) at 1.0 ng/mL (final concentration) were used as internal standards. The samples were sonicated for 3 min in an ultrasonic bath and centrifuged at 15,500 *g* for 10 min at 4°C; then, the supernatants were collected and injected for LC-MS/MS analysis.

The LC-MS/MS analysis was carried out using high-performance liquid chromatography and LC-8060 coupled to a quantum triple quadrupole mass spectrometer (SHIMADZU, Kyoto, Japan). Then, 1 μL of each sample was injected into an InertSustain Swift C8 PEEK column (150 mm, 2.1 mm i.d., 3 μm particle size; GL Science, Tokyo, Japan) at a column temperature of 45°C. For the mobile phase, we used MilliQ water acidified with 0.3% formic acid (Wako Pure Chemical Industries) as solvent A, and acetonitrile (LC-MS/MS grade; Wako Pure Chemical Industries) acidified with 0.3% formic acid as solvent B. Separation of the analytes was achieved using a 12-min binary gradient. After 0.5 min of an isocratic run, the proportion of solvent B was increased over a period of 3 min from 15% to 100%, followed by 4 min at 100% solvent B and then equilibrated at 15% solvent B for the remaining 5 min at a flow rate 0.4 mL/min. We measured the compounds in the electrospray ionization positive ion mode, and the analytical conditions were as follows: the nebulizer gas flow was set to 3.0 L/min, the drying gas flow was set to 8.0 L/min, the heating gas flow was set to 8.0 L/min, the interface temperature was set to 100°C, the desolvation temperature was set to 150°C and the heat block temperature was set to 250°C. Six ceramide species (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0, Cer d18:1/24:0), Sph and dhSph were monitored in the multiple reaction monitoring mode (Table S1), and the data were analyzed using Lab Solution software (SHIMADZU) and standard curves.

Method validation

We obtained Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0, Cer d18:1/24:0, Sph and dhSph from Avanti Polar Lipids. Five concentrations of the standard mixtures of these sphingolipids (0.01, 0.1, 0.5, 1.0 and 10 ng/mL) were prepared in methanol acidified with 0.1% formic acid. These solutions were mixed with 1.0 ng/mL of the internal standard mixture. For intra- and interday precision analysis, we prepared three pooled urine samples by mixing five samples. The precision was evaluated by calculating the coefficient of variation (%).

Urinalysis

The urine sediment examination was carried out using manual microscopy and complied with the Japanese Committee for Clinical Laboratory Standards[31]. As RTEs and granular casts were reported to be involved in tubular injury[32], we evaluated the association between urinary ceramides and renal tubular injury using the sediment score described previously[32]. RTEs were counted per high power field of view, and the casts were counted per whole field. We used the following reagents to measure the clinical urinary markers: total protein (TP; Micro TP-test; Wako Pure Chemical Industries) was measured using the pyrogallol red method; microalbumin

(μAlb; Autowako microalbumin; Wako Pure Chemical Industries) was measured using a turbidimetric immunoassay; *N*-acetyl-β-D-glucosaminidase (NAG; Ltype Wako NAG; Wako Pure Chemical Industries) was measured using an enzyme assay; creatinine (Ltype Wako CRE•M; Wako Pure Chemical Industries) was measured using an enzyme assay; α1-microglobulin (α1-MG; LZtest "Eiken" α1-M; Eiken Chemical, Tokyo, Japan) was measured using latex agglutination turbidimetry; liver-type fatty acid binding protein (L-FABP; NORUDIA L-FABP; SEKISUI MEDICAL, Tokyo, Japan) was measured using latex agglutination turbidimetry; and neutrophil gelatinase-associated lipocalin (NGAL; U-NGAL Abbott; Abbott, Tokyo, Japan) was measured using a chemiluminescence immunoassay. TP, μAlb, NAG, α1-MG and L-FABP were quantified using a 7180 analyzer (Hitachi High-Technologies, Tokyo, Japan), and NGAL was quantified using an ARCHITECT i1000SR (Abbott).

Statistical analysis

The data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). To compare the values among the stages of diabetic nephropathy, we carried out the Kruskal–Wallis test followed by the Steel–Dwass test as a post-hoc test, and to investigate the correlations, we used Spearman's correlation test, as normality or equality of variance had been rejected with the Kolmogorov–Smirnov test or the Bartlett test for most of the analyses. We evaluated the independent effects of the ceramide species on the urine sediment score using a stepwise multiple linear regression analyses with Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0, Cer d18:1/24:0, TP, μAlb, NAG, α1-MG, L-FABP and NGAL as possible explanatory variables. *P*-values <0.05 were deemed statistically significant for all the analyses.

RESULTS

Development of LC-MS/MS detection system for ceramide and Sph species

Extracted ion chromatograms achieved from the multiple reaction monitoring transitions are shown in Figure S1. The presence of urine extract caused the mean of ion enhancement (standard deviation) of 117.6% (8.7%), 139.5% (19.5%) and 112.9% (3.3%) for Sph, dhSph and Cer d18:1/17:0, respectively (Figure S2a). To investigate the influence of albumin on the measurement of urinary ceramide and Sph species, we mixed 0.1% or 1.0% albumin with urine specimen, and measured the levels of ceramides and Sphs. As shown in Figure S2b, we observed no significant influences. We defined the lower limit of quantification as the lowest concentration of the standard compounds that was detected with a signal-to-noise ratio of >10. The results of the calibration curves for the sphingolipids are shown in Figure S3. The curves were fitted to a linear equation of the slope and intercept. Correlation coefficients were better than 0.999 for all the analytes. The results for intraday and interday precision are shown in Table S2. Although DhSph was not detected and the interday coefficient of variation for Cer d18:1/18:1 exceeded 20%, both the intraday and interday coefficients of variation were <20% for the other molecules.

Urinary levels of ceramides were higher in participants with stage 3 diabetic nephropathy

The characteristics of the participants are shown in Table 1. Urinary levels of TP and μAlb were significantly higher in participants with stage 2, 3 and 4 diabetic nephropathy than control participants and those with stage 1 diabetic nephropathy. They were also higher in the participants with stage 3 and 4 diabetic nephropathy than those with stage 2 diabetic nephropathy. Urinary NAG levels were higher in the

Table 1 | Characteristics of the participants

DN stage	Non-DM (*n* = 15)	1 (*n* = 28)	2 (*n* = 24)	3 (*n* = 8)	4 (*n* = 4)	*P*-value
Age (years)	59.9 ± 14.1	65.8 ± 11.1	67.4 ± 11.9	63.3 ± 19.1	65.5 ± 5.8	0.510
Sex						
Male	5	17	16	6	2	0.228
Female	10	11	8	2	2	
eGFR	73.8 ± 10.1	73.6 ± 15.2	60.3 ± 19.2†	55.1 ± 17.5	15.2 ± 10.6*,†,‡	<0.001
HbA1c (%)	5.7 ± 0.4	7.6 ± 1.2*	7.7 ± 1.2*	8.0 ± 1.5*	7.0 ± 0.5*	<0.001
Urinary markers						
TP [g/gCr]	0.06 ± 0.04	0.06 ± 0.04	0.18 ± 0.10*,†	3.49 ± 4.34*,†,‡	2.54 ± 1.83*,†,‡	<0.001
μAlb (mg/gCr)	9.4 ± 6.8	12.5 ± 7.0	92.9 ± 58.4*,†	2393.2 ± 3034.4*,†,‡	1528.5 ± 1103.4*,†,‡	<0.001
NAG (U/gCr)	2.9 ± 1.3	7.6 ± 6.7*	10.1 ± 9.4*	18.6 ± 10.4*,†	10.4 ± 3.9*	<0.001
α1-MG (mg/gCr)	4.1 ± 2.9	5.9 ± 3.9	13.7 ± 13.7*	26.9 ± 22.7*,†	86.8 ± 65.1*,†,‡	<0.001
L-FABP (μg/gCr)	2.1 ± 1.1	2.2 ± 1.9	7.7 ± 8.4†	72.6 ± 115.4*,†	167.7 ± 154.2*,†,‡	<0.001
NGAL (μg/gCr)	15.8 ± 17.9	17.2 ± 15.3	44.7 ± 90.1	130.45 ± 152.9	607.5 ± 643.1*,†	<0.001

*$P < 0.05$ versus non-diabetes (DM). †$P < 0.05$ versus stage 1. ‡$P < 0.05$ versus stage 2. α1-MG, α1-microglobulin; μAlb, microalbumin, eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; L-FABP, liver-type fatty acid binding protein; NAG, *N*-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; TP, total protein.

participants with diabetes than those without diabetes, and higher in stage 3 diabetic nephropathy than stage 1 diabetic nephropathy. The levels of α1-MG were significantly higher in stage 2, 3 and 4 diabetic nephropathy than control participants, and were higher in stage 3 and 4 diabetic nephropathy than stage 1 or 2 diabetic nephropathy. L-FABP levels were higher in stage 3 and 4 diabetic nephropathy than control participants, and also higher in stage 2, 3 and 4 diabetic nephropathy than stage 1 or 2 diabetic nephropathy. NGAL levels were significantly higher in stage 4 diabetic nephropathy than control participants and those with stage 1 diabetic nephropathy.

First, we compared the urinary levels of ceramides and Sph according to the stage of diabetic nephropathy. As a result, the urinary Sph and Cer d18:1/16:0 levels were significantly higher in the participants with stage 1, 2 and 3 diabetic nephropathy than control participants (Figure 1a,b). We also observed that Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0 levels were significantly higher in participants with stage 3 diabetic nephropathy than normal participants (Figure 1b–g). In regard to the ratios of specific ceramide classes to other ceramide classes, the ratio of C18:0, C20:0, C22:0 and C24:0 to C16:0 were significantly low in patients with stage 1, 2 and 3 diabetic nephropathy (Figure S4a–d), and the ratio of C20:0 to C18:0 and that of C24:0 to C22:0 were significantly higher in stage 1 and 2 diabetic nephropathy than control participants (Figure S4e,j). The ratio of C22:0 to C20:0 was significantly lower in stage 1 and 2 diabetic nephropathy than control participants, and higher in stage 3 than stage 2 diabetic nephropathy (Figure S4h). Other ratios of ceramides were not significantly modulated. All the ceramide species and Sph were correlated with each other in a positive manner (Figure S5); in particular, a strong positive correlation was observed between long-chain ceramides, such as C16:0 and C20:0 ($r = 0.848$, $P < 0.001$), and between very long-chain ceramides, such as C22:0 and C24:0 ($r = 0.824$, $P < 0.001$).

Urinary levels of ceramides were positively correlated with urinary clinical biomarkers

Next, we compared the urinary levels of ceramides or Sph and urinary clinical biomarkers. We observed that almost all of ceramides and Sph levels were positively correlated with urinary biomarkers (Table S3). In particular, Cer d18:1/24:0 was moderately correlated with TP ($r = 0.565$, $P < 0.001$), μAlb ($r = 0.495$, $P < 0.001$), NAG ($r = 0.548$, $P < 0.001$), α1-MG ($r = 0.478$, $P < 0.001$), L-FABP ($r = 0.477$, $P < 0.001$) and NGAL ($r = 0.436$, $P < 0.001$). As representative results, the correlations between Cer d18:1/24:0 and biomarkers are shown in Figure 2.

Urinary levels of ceramides were higher in participants with sediment score 2

In addition to urinary chemical biomarkers, we investigated the association between urinary sphingolipids and the results of a urine sediment test, using the sediment score[32]. As a result, Sph, Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0 were significantly higher in score 2 than score 1 (Figure 3). When we investigated the significant explanatory factors for the sediment score using a logistic regression analysis, we found that Cer d18:1/20:0 and TP was extracted as a significant predictor of score 2 (Table S4).

DISCUSSION

In the present study, we developed a simultaneous quantitative measurement system for ceramides and Sphs in urine specimens. Using this method, we next investigated the urinary levels of ceramides and Sphs in patients with diabetic nephropathy. We found that the levels of Sph and Cer d18:1/16:0 were higher in patients with early-stage diabetic nephropathy than control participants, and that Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0 were higher in patients with stage 3 diabetic nephropathy (Figure 1). Furthermore, the urinary levels of ceramides were positively correlated with urinary clinical biomarkers (Figure 2) and the sediment score (Figure 3).

Although the urinary levels of ceramides were much lower than those in serum, linear relationships between Sph and six kinds of ceramides were observed up to a low concentration of 0.01 ng/mL and up to a concentration of 0.1 ng/mL for dhSph using our method. In clinical specimens, good precision was obtained for Cer d18:1/16:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0. Compared with previous reports, we were able to reduce the measurement time and improve the sensitivity while maintaining the same precision[26,33].

Regarding the urinary levels of ceramides and Sph in patients with diabetic nephropathy, we observed that the levels of all the ceramide species except for Cer d18:1/18:1, the concentration of which was relatively low in urine samples, were higher in the participants with stage 3 diabetic nephropathy, whereas the urinary levels of ceramides were not higher for patients with stage 4. Although the reason for the decrease in urinary ceramides at stage 4 remains unclear, the decrease might be related to the progression of kidney injury and reductions in the synthesis and/or excretion of ceramides from the kidney. Although Magagnotti *et al.* very recently reported that the urinary ceramide levels were higher in 15 children with type 1 diabetes than in 35 healthy children, the present study is the first study to show associations between urinary ceramides and the stages of diabetic nephropathy or clinical parameters for diabetic nephropathy in adults. As for the classes of ceramides, previous studies have reported that the plasma Cer C18:0, C20:0 C22:0, C24:1 and dihydro Cer C22:0 levels were higher in patients with diabetes[9–11], with similar changes in the urinary levels of ceramides observed in the present study. Hilvo *et al.* reported that the plasma C18:0/C16:0 ratio is an independent predictive biomarker for incident diabetes[11]. In the present study, however, the urinary C18:0-to-C16:0, C20:0-to-C16:0, C22:0-to-C16:0, C24:0-to-C16:0 and C22:0-to-C20:0 ratios were significantly lower in the diabetes patients than the control

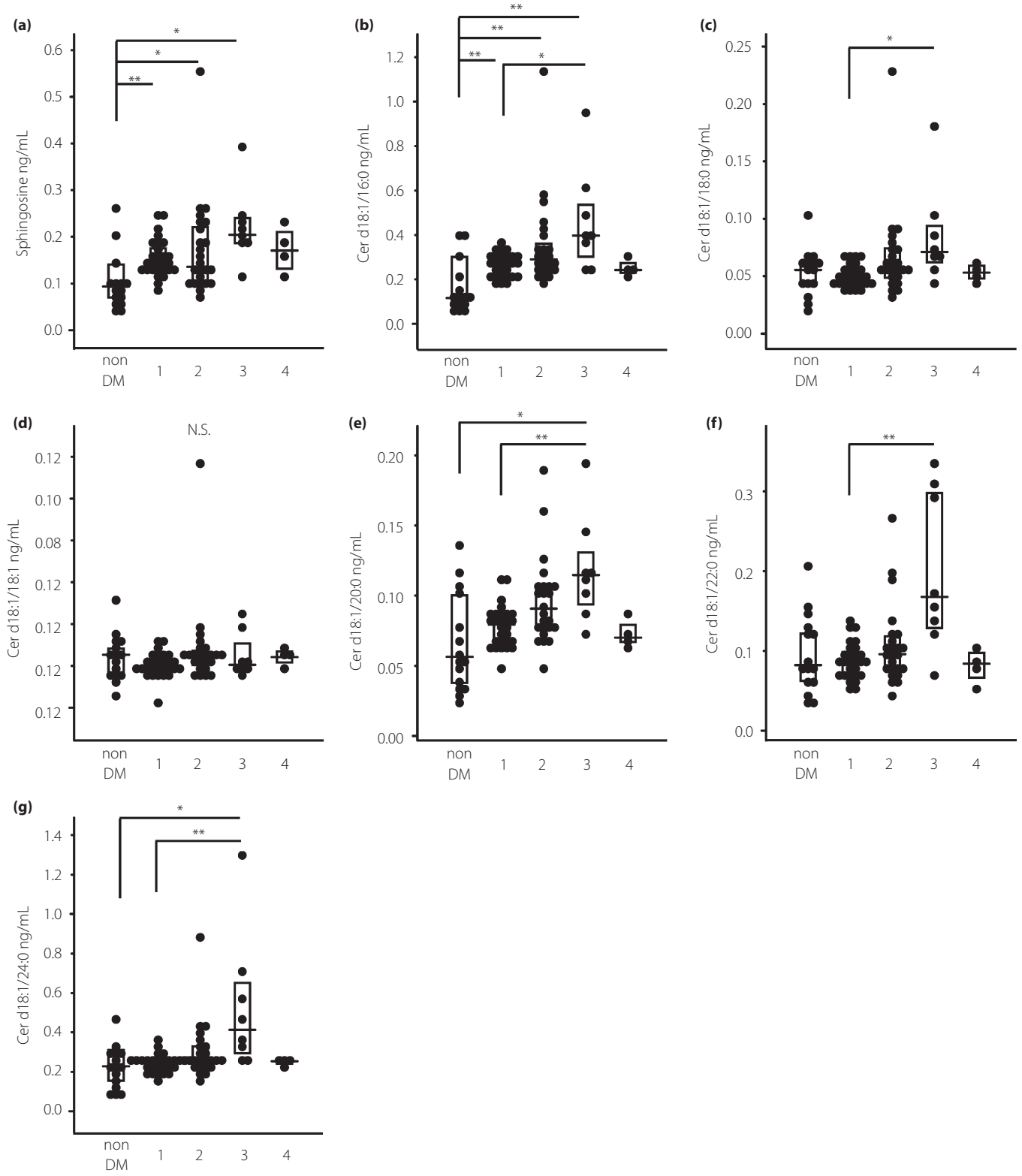

Figure 1 | Increased levels of urinary ceramides in patients with stage 3 diabetic nephropathy. Urinary sphingosine and ceramide species were measured in participants with and without type 2 diabetes. (a) Sphingosine, (b) Cer d18:1/16:0, (c) Cer d18:1/18:0, (d) Cer d18:1/18:1, (e) Cer d18:1/20:0, (f) Cer d18:1/22:0 and (g) Cer d18:1/24:0, as determined using liquid chromatography-tandem mass spectrometry. *$P < 0.05$, **$P < 0.01$. DM, diabetes.

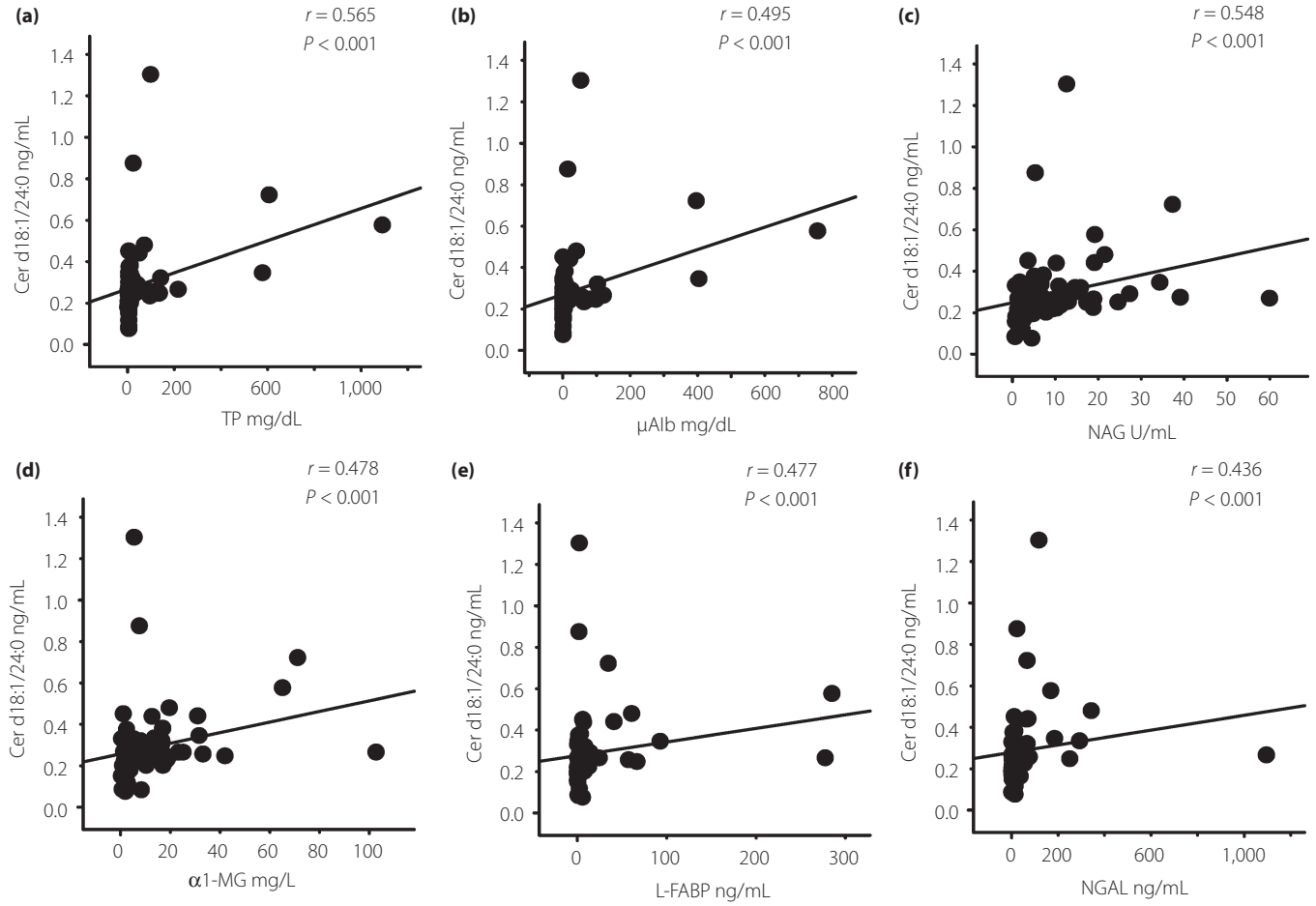

Figure 2 | Correlations between urinary ceramide and chemical biomarkers. The correlations between urinary ceramide and chemical biomarkers were investigated. (a–f) Correlations between Cer d18:1/24:0 and (a) total protein (TP), (b) microalbumin (μAlb), (c) *N*-acetyl-β-D-glucosaminidase (NAG), (d) α1-microglobulin (α1-MG), (e) liver-type fatty acid binding protein (L-FABP) and (f) neutrophil gelatinase-associated lipocalin (NGAL) are shown. The correlations between these biomarkers and other ceramide species are shown in the Supplemental Data.

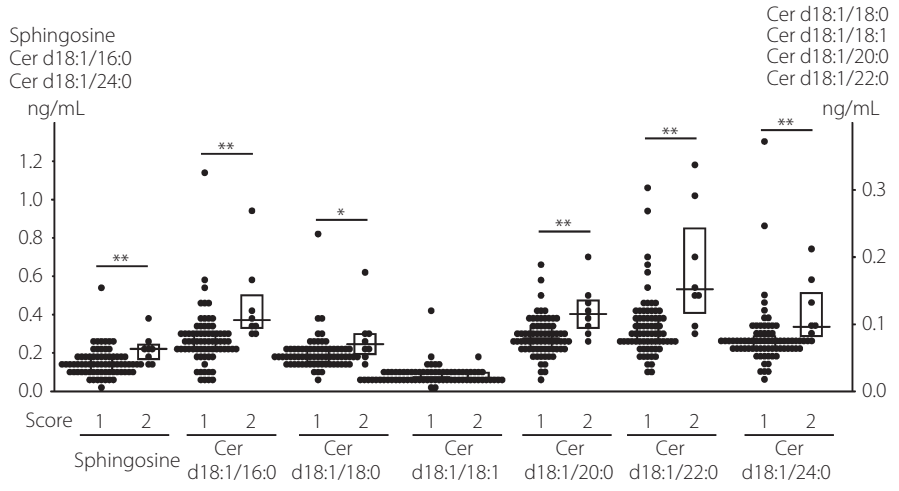

Figure 3 | Association between urinary sphingolipids and sediment score. Urinary sphingosine and ceramide levels were compared with the sediment score. There were no samples classified as score 3 among the participants. *$P < 0.05$, **$P < 0.01$.

participants (Figure S4), whereas the C22:0-to-C20:0 ratio was higher in patients with stage 3 diabetic nephropathy. These findings suggest that the modulation of ceramide metabolism by diabetic nephropathy might differ between serum and urine, and that the modulation of urinary ceramide levels by diabetic nephropathy might be different depending on ceramide classes; urinary Cer C16:0 levels might be modulated by the pathogenesis of early diabetic nephropathy[34], and Cer C22:0 levels might be influenced by the progressed diabetic nephropathy. One of the mechanisms for this unique modulation of very long-chain ceramide species in urine might involve the class of ceramide synthase (CerS) expressed in the kidney. CerS is one of the causes of ceramide fluctuation, and among CerS classes, CerS2 is highly expressed in the kidney and has the highest proportion of C22 to C24 ceramides[35]. Considering our finding that the urinary levels of Cer C22:0 and C24:0 were higher in patients with stage 3 diabetic nephropathy, these urinary ceramides might be mainly derived from the kidney. Actually, a CerS2 variant reportedly elevates the risk of an increase in albuminuria[36], although whether CerS2 function directly causes albuminuria remains controversial; no phenotypic abnormalities in the kidney have been reported in CerS2-deficient mice[37]. Further basic studies are required to show the involvement of CerS2 in the pathogenesis of diabetic nephropathy.

Furthermore, the present study showed that urinary ceramides were correlated with not only TP and μAlb, but also with chemical biomarkers of injury, such as NAG, α1-MG and L-FABP. These results suggest that ceramides might be associated with renal tubular injury. Actually, ceramides were significantly and positively associated with the number of RTEs and granular casts based on the sediment score, which are also indicators of tubular injury (Figure 3). In individuals with diabetic nephropathy, renal function declines and structural tubular injury progresses, and some studies using animal models have reported increased levels of serine palmitoyl transferase or ceramide synthase in RTEs,[18] and that the intracellular ceramide level increased in response to oxidative stress in RTEs[38], as supported by the results of the present study.

The main limitation of the present study is that as this was an observational study, whether the changes in urinary ceramides are a cause or result of kidney injury remains uncertain. Further analyses using animal models or clinical studies investigating the prognosis or incidence of diabetic nephropathy are required to resolve this issue. Another limitation is that we examined a rather small number of patients with stage 4 diabetic nephropathy. We observed that the urinary levels of ceramides were lower in patients with stage 4 diabetic nephropathy than in those with stage 3, and these changes were consistent with chemical biomarkers reflecting tubular injury.

In summary, our method is useful for the measurement of urinary Sph and ceramides in clinical specimens, and the present study suggested the possible association between urinary levels of ceramides and the pathological conditions of kidney diseases, such as renal tubular injury.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Retention time and chromatograms of sphingolipids and ceramides.

Figure S2 | Matrix effects and influence of urinary albumin on the measurement.

Figure S3 | Calibration curves of analytes. The calibration curves for sphingolipids and ceramides are shown. The calibration range was (a) 0.01–10 ng/mL for sphingosine (Sph), (b) 0.1–10 ng/mL for dihydrosphingosine (dhSph) and (c–h) ceramide species.

Figure S4 | The levels of urinary ceramides and the ratio of very long-chain ceramides to long-chain ceramides. Urinary sphingosine and ceramide species were measured in participants with or without diabetes.

Figure S5 | Correlations between urinary ceramide species. The correlations between very long-chain (C22–24) ceramides and long-chain (C16-20) ceramides were investigated.

Table S1 | Multiple reaction monitoring settings for liquid chromatography-tandem mass spectrometry

Table S2 | Precision of the simultaneous measurement system for urinary sphingolipids

Table S3 | Correlation between urinary sphingolipids and ceramides and other markers

Table S4 | Logistic regression analysis for the sediment score