Review

Urinary lipid mediators as novel biomarker for kidney disease

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Abstract Bioactive lipids, including lysophospholipids, sphingolipids, eicosanoids, and their metabolites, hold promise as potential biomarkers for various diseases. Recent advances in liquid chromatography-tandem mass spectrometry (LC-MS/MS) have enabled accurate quantification of these lipids. Our research focuses on applying the LC-MS/MS methods developed by us to urine samples for diagnosing non-invasive diseases. Urine, is easier to collect and is less variable than blood, and provides a suitable matrix for the detection of bioactive lipids. Significant changes have been identified in urinary lipid mediators in various kidney diseases such as diabetic nephropathy, chronic glomerulonephritis, and systemic lupus erythematosus. These findings indicate that urinary lipid profiles can serve as biomarkers for disease progression and severity. Moreover, the use of machine learning models in conjunction with lipidomics has further enhanced the accuracy of differentiating kidney diseases. Despite challenges such as equipment complexity and data management, mass spectrometry in conjunction with artificial intelligence technologies offers promising new diagnostic approaches. Ultimately, profiling lipid mediators in urine specimens could revolutionize disease understanding and clinical laboratory testing.

Key words: urine, lysophospholipids, ceramide, sphingolipids, liquid chromatography-tandem mass spectrometry

Introduction

Recent studies have demonstrated bioactive lipids including lysophospholipids (lysoPLs)[1–4], sphingolipids[5–7], eicosanoids, and docosanoids[8–11], to be promising biomarkers for diagnosing various human diseases. Derived from membrane phospholipids, these lipids play various physiological roles. Bioactive lipids are found in various molecular species[12–14]. They differ with respect to the lengths of the fatty acid carbon chains and the number of unsaturated bonds, and each molecular species potentially has different bioactivities. Comprehending the functions of these lipids can elevate our knowledge of pathophysiology and contribute to the development of new therapies and diagnostic methods.

Recent advances in analytical techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), have enabled the simultaneous and accurate quantification and identification of multiple molecular species of bioactive lipids[15–19]. There are two possible approaches in mass spectrometry (MS): "target analysis," optimizes system parameters for known analyte compounds and quantifies them, and "non-target analysis," identifies compounds by comparing unknown peaks obtained in measurements with a database. MS technology has already been applied in clinical tests in various fields including newborn mass screening[20–22], identifying infectious disease-causing microorganisms[23, 24], hormone and lipid analysis[25], and drug and toxin analysis[26, 27]. Recent enrichment in databases, has made the discovery of biomarkers using shotgun lipidomics analysis and shotgun proteomics analysis increasingly common[28–31].

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With regards to the analysis of bioactive lipids in human specimens, this study has focused on applying LC-MS/MS method on urine specimens. Urine samples are easy and non-invasive to collect. Urinalysis has long been used as a screening test for kidney and urinary tract diseases. Besides, since LC-MS/MS enables the simultaneous multi-component analysis with high sensitivity and specificity, and enables the detection of trace substances in samples, LC-MS/MS can be a suitable method for analyzing bioactive lipids in urine specimens.

We hereby present the findings of our study that indicate that the modulations of bioactive lipids in urine samples can enable the diagnosis of various diseases and provide future directions for applying LC-MS/MS in practical clinical laboratory testing.

Urine is a suitable human specimen for measuring bioactive lipids

With regards to human specimens for analyzing bioactive lipids, strict sampling is required for blood samples when measuring lysophosphatidic acid and sphingosine-1-phosphate (S1P) in biological samples, due to the fluctuations caused by the presence of blood components post-collection[32]. However, urine samples are considered to reflect pathological states more sensitively since they have fewer blood and plasma components and lower concentrations of bioactive lipids under healthy conditions. In this study, the effects of incubation on levels of eicosanoids and their metabolites (Fig. 1A), and lysoPLs (Fig. 1B) were investigated using urine specimens obtained from healthy controls, as well as from individuals with pyuria and hematuria. As evident from Fig. 1A, the urinary levels of eicosanoids and their metabolites (representative data has been depicted), including tetranor-prostaglandin E metabolite (tetranor-PGEM), Maresin 1, and docosahexaenoic acid (DHA), were relatively low and did not undergo significant changes over time in healthy controls. After 24 h of incubation, the levels of tetranor-PGEM in pyuria and hematuria samples decreased and that of DHA in pyuria sample increased (Fig. 1A)[33]. As shown in Fig. 1B, the levels of urinary lysoPLs were relatively low and stable in healthy controls. However, the total levels of lysoPC, lysoPE, and lysoPS in pyuria samples, as well as lysoPC, lysoPE, and lysoPG in hematuria samples, increased after 24 h of incubation (Fig. 1B).

These results demonstrate that urinary bioactive lipids are relatively stable in healthy individuals but show significant changes in response to pathological conditions such as pyuria and hematuria. This stability under normal conditions, coupled with the ability to detect alterations associated with disease, suggests that urine can effectively reflect changes in bioactive lipid levels. However, to avoid inaccurate results due to lipid level increases from metabolic processes, rapid measurement of urine samples is crucial. Thus, urine is a suitable and reliable specimen for the measurement of bioactive lipids. Its stability in healthy states and sensitivity to pathological changes make it a valuable alternative to blood for assessing bioactive lipid profiles in clinical research and diagnosis.

Urinary bioactive lipids in chronic kidney diseases

In this study, we have established and reported clinically useful measurement methods for quantifying lysoPLs[34], sphingolipids (such as ceramides and S1P)[35–37], and eicosanoid/omega-3 fatty acid metabolites in urine samples using LC-MS/MS[19, 33]. The comprehensive analysis methods and their validations have been detailed in previous reports[33–37]. Briefly, urine specimens were mixed with methanol acidified with formic acid, and the supernatant was quantified using an internal standard method. For the pretreatment of eicosanoids and their metabolites, lipid extraction was performed using solid-phase extraction procedures[33]. The LC-MS/MS analysis was carried out using high-performance liquid chromatography and LC-8060 coupled to a quantum triple quadrupole mass spectrometer. Each method was evaluated based on matrix effects, reproducibility, and linearity.

First, using the methods for measuring ceramides and sphingosine, we focused on their urinary levels in the patients with diabetic nephropathy (DN). We have highlighted that significant elevation of urinary sphingolipids in stage 3 nephropathy makes measurement of urinary sphingolipids significant in DN. This also indicates the involvement of urinary sphingolipids in the pathophysiology of DN. Among various ceramides, level of C16:0 ceramide was observed to have elevated from the early stages of DN, while that of C22:0 ceramide was elevated in stage 3 of DN, indicating possible differences in pathophysiology by molecular species. Furthermore, analyses of the correlation between urinary sediment tests and tubular injury biomarkers indicate that ceramides reflect tubular injury[36].

We also found that the simultaneous analyses of urinary

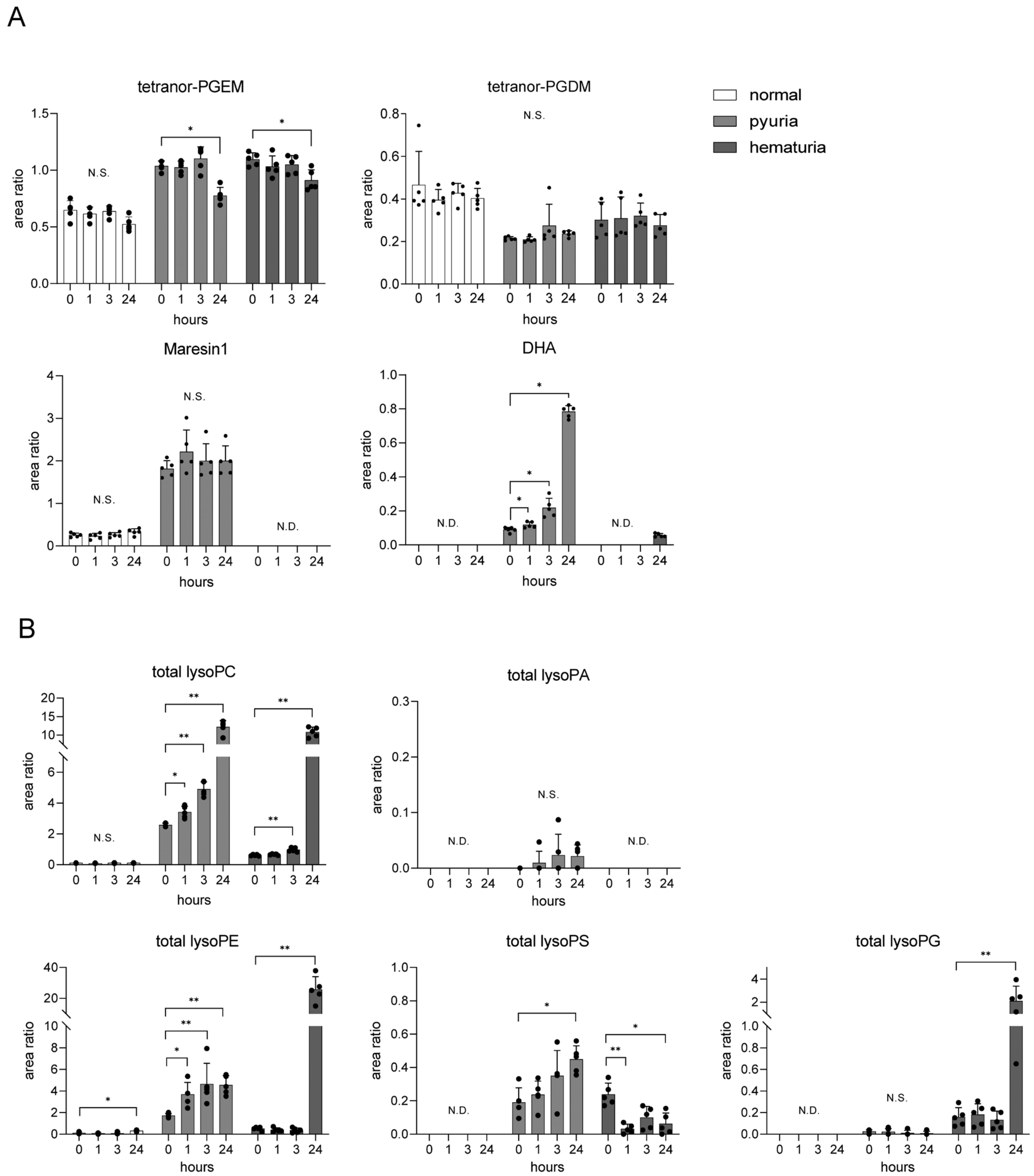

Fig. 1. Effects of incubation on urinary lipid metabolites.

The effects of incubation on the measurement of urinary eicosanoids and related metabolites: (A) and lysophospholipids (B) using three samples: normal (white bar), pyuria (light gray bar), and hematuria (gray bar). Each sample was incubated for 0, 1, 3, and 24 h; The metabolites that were detected in the urinary samples have been shown. Data are shown as the mean±SD of the peak area ratio (n=5); $^{*}P<0.05$ and $^{**}P<0.01$ versus 0 h, N.S.: no significant; N.D.: not detected; SD: standard deviation. Fig. 1A is taken from Reference 33 and partially modified.

Abbreviations: tetranor-PGEM: tetranor-prostaglandin E metabolite; tetranor-PGDM: tetranor-prostaglandin D metabolite; DHA: docosahexaenoic acid; lysoPC: lysophosphatidylcholine; lysoPA: lysophosphatidic acid; lysoPE: lysophosphatidylethanolamine; lysoPS: lysophosphatidylserine; lysoPG: lysophosphatidylglycerol.

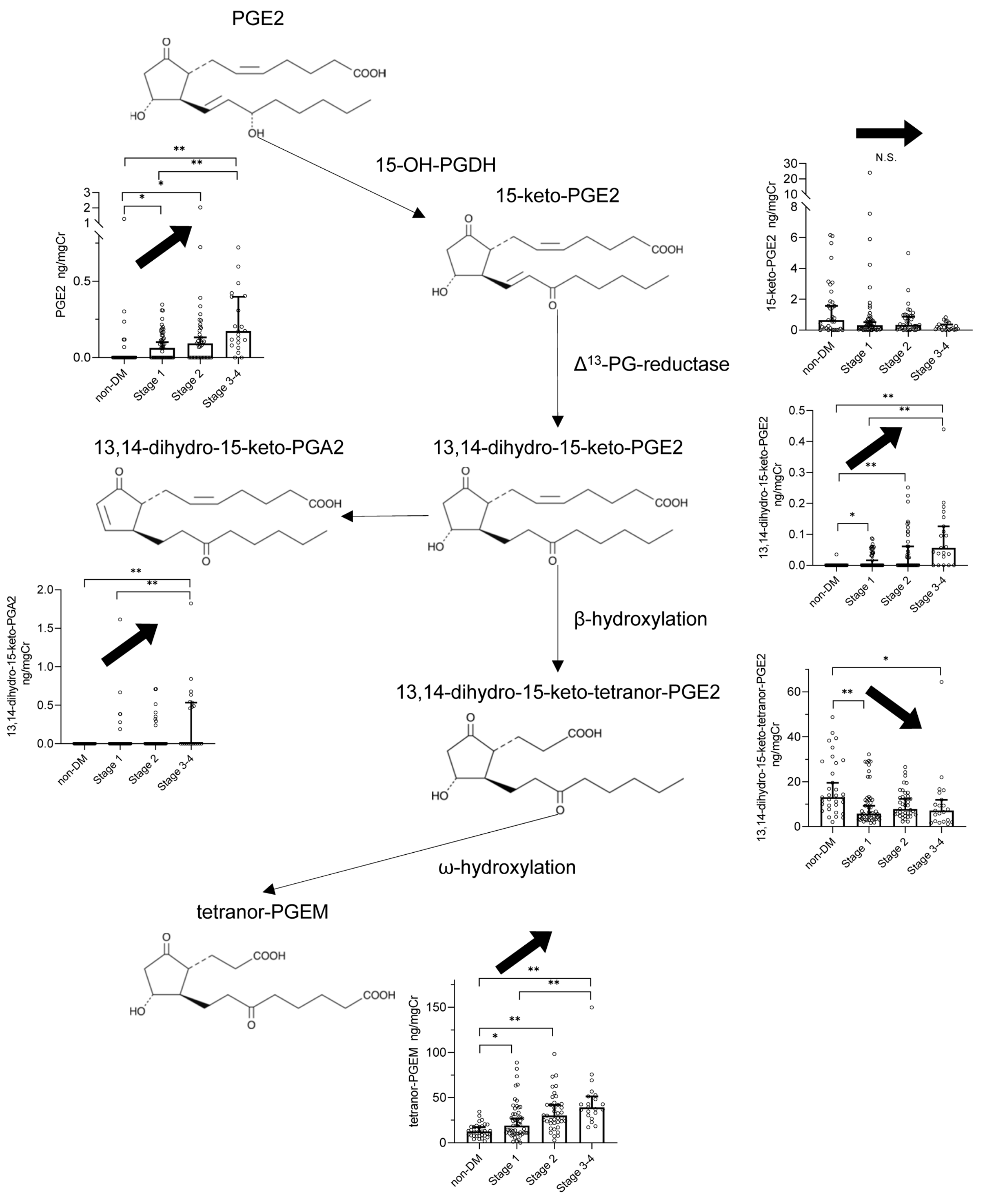

Fig. 2. Schematic figure of pathway of prostaglandin E metabolites and variations in their levels in diabetic nephropathy.
The metabolic pathway of prostaglandin E (PGE) has been shown. Black arrows indicate the modulation of urinary PGE2 and its metabolites in the subjects with diabetic nephropathy. Fig. 2 has been taken from Reference 33 and has been partially modified.

eicosanoids, leukotrienes, and their metabolites indicated tetranor-PGEM to be a possible novel biomarker for DN[33]. Patients with DN had significantly elevated levels of urinary tetranor-PGEM and these patients had more advanced stages of DN, indicating the potential of urinary tetranor-PGEM as a marker for disease progression. Besides, the LC-MS/MS method that we developed, enabled us to profile the related urinary PGE2 metabolites (Fig. 2).

In addition to patients suffering from diabetes mellitus (DM), urinary sphingolipids were also analyzed in patients

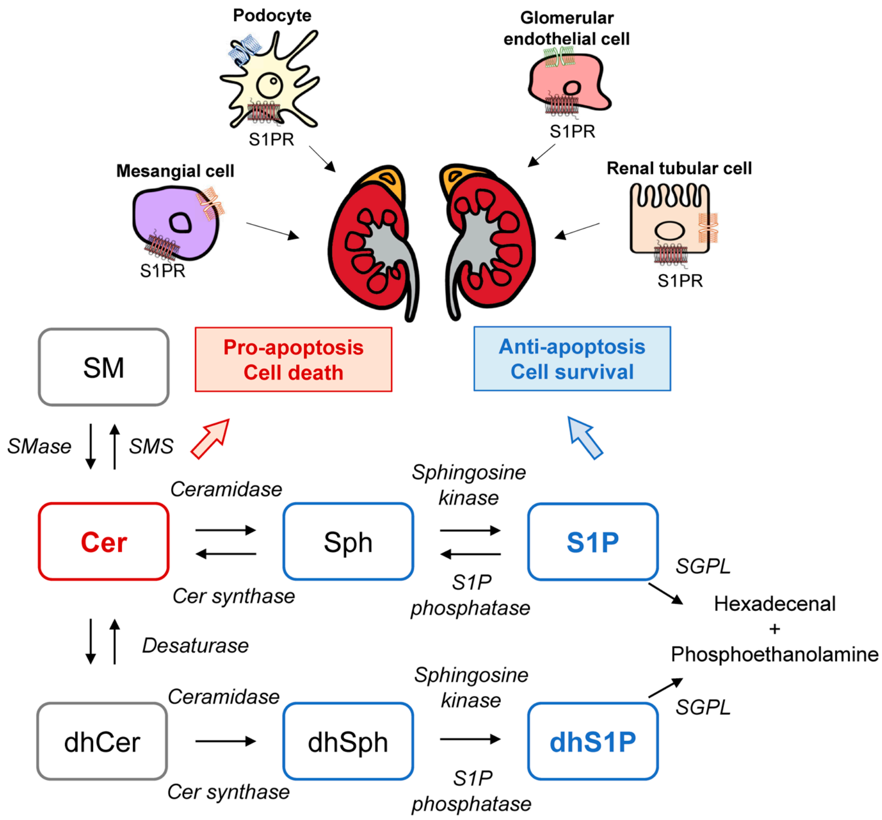

Fig. 3. Schematic figure of the sphingolipid metabolism in kidney.

Ceramide is involved in apoptosis, inflammation, and cell death, while S1P and dhS1P have roles in anti-apoptosis and cell survival. S1P receptors are expressed in renal mesangial cell, podocyte, glomerular endothelial cell, and tubular cell and play an important role in the sphingolipid metabolism in kidney.

Abbreviations: Cer: ceramide, S1P: sphingosine-1-phosphate, Sph: sphingosine, dhSph: dihydrosphingosine, SPHK: sphingosine kinase, SGPL: S1P lyase, SM: sphingomyelin, SMase: sphingomyelinase, SMS: sphingomyelin synthase.

suffering from chronic kidney disease (CKD) including chronic glomerulonephritis (CGN), arterial hypertension (AH), and systemic lupus erythematosus (SLE). CKD is a long-term condition where the kidneys gradually lose function. Although renal biopsy is sometimes required to diagnose kidney diseases, it is invasive procedure. Therefore, developing novel noninvasive laboratory tests for the differential diagnosis of kidney diseases remains an important task. Ceramide is reportedly involved in apoptosis, inflammation, and cell death[38)], while S1P and dhS1P play a role in anti-apoptosis and cell survival (Fig. 3)[39–42)]. The results demonstrated the urinary S1P was higher in patients with DM than in other groups, whereas dihydroS1P was lower in the CGN and AH groups compared with control group. Sphingosine and dihydrosphingosine were higher in the CGN, AH, and SLE groups than in control group (Fig. 4A). The urinary ceramides were significantly higher in the CGN, AH, and SLE groups than in control and DM groups (Fig. 4B). These findings indicate that the variations in urinary sphingolipids differ depending on the disease[43)].

Urinary glycerophospholipids and sphingolipids in diseases complicated by renal impairment

Glycerophospholipids and sphingolipids might be linked to both acute and chronic kidney diseases due to their bioactive functions. We hypothesized that pathophysiological condition associated with renal complications might alter the levels of urinary glycerophospholipids and sphingolipids. Consequently, we investigated the variations in the urinary bioactive lipids in the patients with COVID-19, as these patients often suffer from renal complication (COVID-19-associated kidney injuries). COVID-19 patients were categorized into three groups based on disease severity: Those who did not require oxygen supplementation, those who required oxygen but not mechanical ventilation, and those who required mechanical ventilation. Significant modulations were observed in the urinary levels of sphingolipids and glycerophospholipids in COVID-19 patients

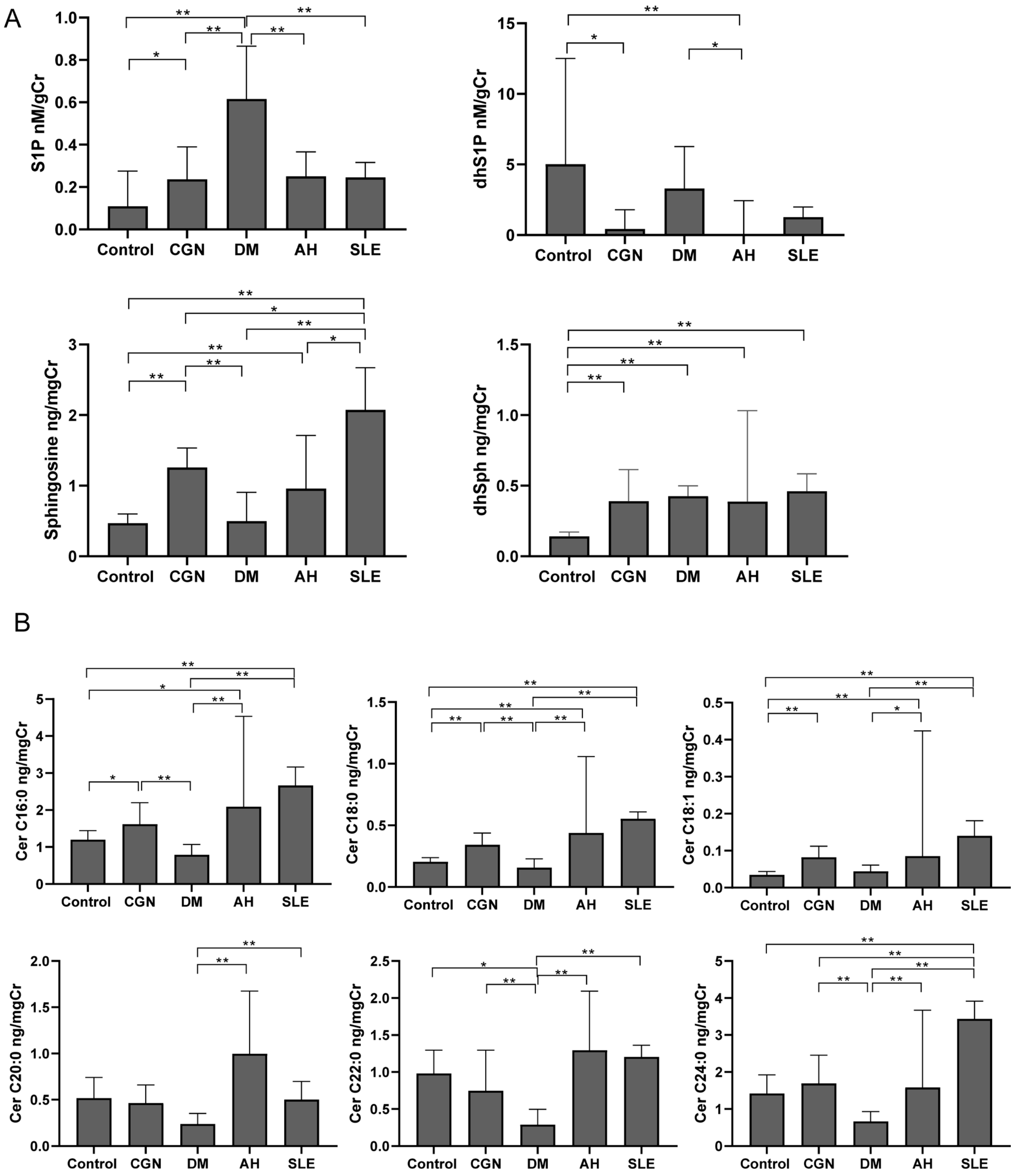

Fig. 4. Modulations of the urinary sphinogolipids among kidney diseases.

The urinary levels of (A) sphingosine 1-phosphate (S1P), dihydroS1P (dhS1P), sphingosine, dihydrosphingosine (dhSph), and (B) ceramides C16:0, C18:0, C18:1, C20:0, C22:0, and C24:0 by kidney disease type—chronic glomerulonephritis (CGN), diabetes mellitus (DM), arterial hypertension (AH), and systemic lupus erythematosus (SLE)—are shown. Data are shown as the median± 95% confidence interval. $^{*}P<0.05$, $^{**}P<0.01$. Figure 4 has been taken from reference 43 and has been partially modified.

compared to controls. Notably, C16:0 Cer exhibited the most rapid increase between days 4 and 6. Conversely, C18:1 Cer, C20:0 Cer, C22:0 Cer, C24:0 Cer, Sph, dhSph, and dhS1P showed significant decreases or a decreasing trend during the early phase (days 1−3) in the maximum severity group 1, before rising again, with a notable increase in maximum severity group 3 (see Fig. 1 in reference 44). Among the sphingolipids monitored, C18:0 Cer levels declined quickly from days 1 to 3. Regarding monitored glycerophospholipids, including lysophosphatidylcholine (lysoPC), lysophosphatidic acid (lysoPA), lysophosphatidylserine (lysoPS), lysophosphatidylethanolamine (lysoPE), lysophosphatidylglycerol (lysoPG), lysophosphatidylinositol (lysoPI), as well as their corresponding phospholipids, exhibited increased levels, with a particularly notable rise in severe COVID-19 cases (see Fig. 2 in reference 44). The relationship between these alterations in lipid levels and acute kidney injury in COVID-19 patients was also explored. Elevated levels of specific ceramides and reduced levels of S1P were associated with kidney injury, indicating that these lipids could be used as biomarkers for COVID-19-associated renal damage[44)]. The findings indicate that disease progression and kidney injury in COVID-19 patients can be monitored through urinary lipid profiles. This could lead to better clinical management and potentially new therapeutic targets aimed at mitigating kidney damage caused by the virus. The study highlights the significance of lipidomics in deciphering complex biochemical changes in COVID-19 and their impacts on different organ systems.

Urinary bioactive lipids in diseases unrelated to renal impairment

Sugiomoto et al. had previously demonstrated a significant modulation of urinary lipid mediators in acute bladder cystitis[45)]. The elevated levels of prostaglandins and leukotrienes in the urine of patients suffering from cystitis highlight the potential role of these lipid mediators in mediating inflammatory response during acute bladder infections. These lipid mediators might contribute to symptoms such as pain and urinary urgency experienced by these patients. The study also highlights the significance of other lipid mediators such as sphingolipids, which might influence cellular signaling pathways involved in bladder epithelial cell responses to bacterial infection. Our findings indicate the possible role of specific lipid mediators as biomarkers for the diagnosis of bladder infections and offer new insights into therapeutic development. Targeting these lipid pathways might help in designing novel treatments that could alleviate inflammatory symptoms and improve patient outcomes.

Artificial intelligence (AI)-based analysis of urinary sphingolipids

Although simultaneous analyses of urinary bioactive lipids were useful, single-component analysis indicated that the bioactive lipid concentrations overlapped among different diseases, and hence could not be used as definitive indicators for distinguishing kidney diseases. Therefore, an attempt at creating a machine learning model to differentiate between kidney diseases based on urinary sphingolipids, using a forementioned AI-based analysis method was made. SPSS Modeler (IBM), a software application that we have previously used, includes algorithms such as neural networks, decision tree analysis (CHAID, Chi-square automatic interaction detection, Random Trees, etc.), support vector machines, regression analysis (logistic regression, discriminant analysis, etc.), and Bayesian networks. It automatically selects the optimal algorithm for creating the model. The sample data were randomly divided into training and test data sets. An improvement in the prediction accuracy and precision was observed when sphingolipids were included as predictor variables compared to using only existing clinical test data (age, sex, urinary protein, urinary sediment test results, etc.)[43)].

Future directions

As described earlier, the LC-MS/MS method developed by our laboratory demonstrated the involvement of urinary lipid mediators in chronic kidney diseases and other pathophysiological conditions. The lipidomics approach using LC-MS/MS and AI technology on urine specimens might contribute to understanding various diseases and has potential benefits in diagnoses and assessment (Fig. 5). Specifically, urinary bioactive lipids might offer advantages over traditional urinary biomarkers. Profiling urinary bioactive lipids enables the evaluation of a patient's pathological condition through simultaneous analysis of multiple components, rather than relying on a single biomarker. For instance, combining urinary sphingolipids with existing urinary biomarkers can improve the prediction accuracy of distinguishing kidney diseases, compared to using existing urinary biomarkers alone (see Fig. 4 in reference 43). Additionally, a pilot analysis of bioactive lipids may lead to the discovery of novel biomarkers for disease diagnosis and prognosis. Previous studies have shown that urinary tetranor-PGEM levels increases as DN progresses and are associated with a decline of eGFR, even though urinary microalbumin remain a better marker than tetranor-PGEM for distinguishing DN stages[33)].

Clinical laboratory tests are the backbone of medical care

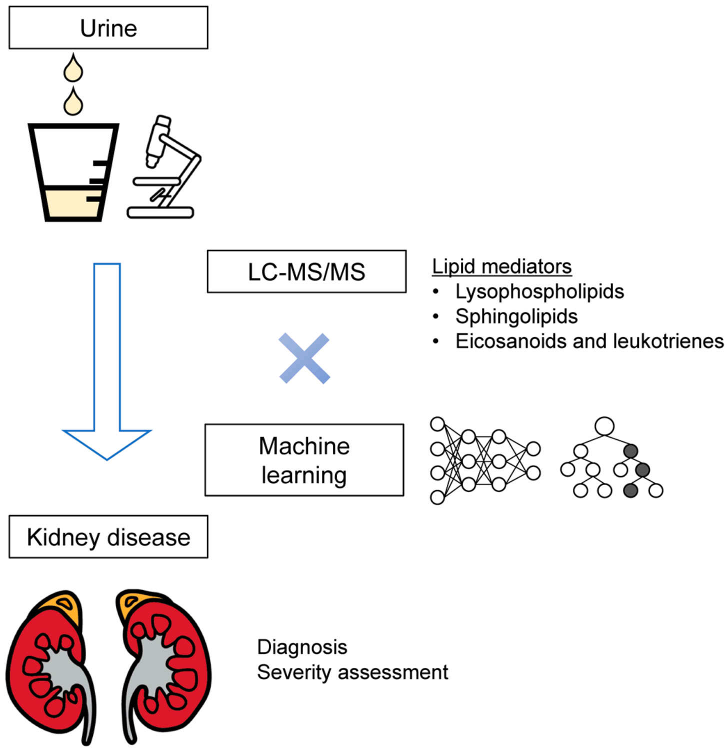

Fig. 5. Perspective for the clinical use of urinary lipid mediators for kidney diseases.
Schematic overview of the study. Utilizing non-invasive and easily collectible urine samples suitable for analysis, lipid mediators were measured using LC-MS/MS. Our objective was to establish a novel testing method for diagnosing kidney disease and evaluating its severity, employing analysis methods that utilize machine learning models.

and play a crucial role as indicators for disease diagnosis, treatment, severity assessment, and prediction of prognosis. Traditionally, clinical tests have focused on developing and evaluating the utility of tests such as tumor markers, that correspond one-to-one with diseases. However, interest in AI is on the rise, and this trend is now influencing clinical laboratory medicine. This has resulted in the current shift toward using AI for big data analysis to identify diseases and pathologies from complex data sets. MS allows simultaneous multi-component analysis, naturally resulting in a large volume of data. AI technology is considered efficient for analyzing such large volumes of data. Recently, terms like "AI," "data science," "big data," and "machine learning" have become prevalent in the field of clinical laboratory medicine[46–48]. This trend can be attributed to advancements in computer performance and software, making them widely and conveniently accessible.

Clinical laboratories contain vast amounts of data, and it would be a waste not to utilize AI technology. In fact, several studies have reported attempts at constructing high-precision automated classification systems using AI for image analysis in applications such as deep learning-based peripheral blood leukocyte classification[49, 50] and automated classification and malignancy determination of cytology and histology images[51, 52]. These studies have shown promising results and have the potential for future clinical application. Moreover, studies incorporating real-world data for analyzing the diagnosis and treatment characteristics of diabetic kidney disease, among others, involving not only clinical laboratory data but also various types of medical information have been reported[53]. In MS, the use of AI technology to integrate and analyze large volumes of data could enable the identification of diseases and pathologies from new perspectives.

Although MS technology has significant potential for clinical laboratory testing, there are several challenges for their effective application in this field. First, the complexity of the equipment and its operation poses a problem. Mass spectrometers are expensive and require specialized knowledge and skills to operate, making its widespread use costly and demanding in terms of training of personnel. Additionally, calibration and maintenance of these devices are labor-intensive. Next, the standardization of sample preparation is crucial. Clinical samples are diverse and cannot often be used directly for analysis. Establishment of simple and standardized protocols is needed as complex pre-treatment processes can affect reproducibility and reliability. Moreover, data analysis and management present significant challenges. MS generated extensive data, that requires advanced techniques for analysis. Proper software and database infrastructure are vital for handling and interpreting these data effectively.

Despite these challenges, the collaboration between MS and AI holds significant potential for novel approaches. Profiling lipid mediators in urine specimens could serve as novel biomarkers for understanding the pathophysiology of diseases.

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Conflict of Interest

All authors have declared the absence of any competing interest.

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