Original Article

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Urine myo-inositol as a novel prognostic biomarker for diabetic kidney disease: a targeted metabolomics study using nuclear magnetic resonance

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Background: As a leading cause of chronic kidney disease, clinical demand for noninvasive biomarkers of diabetic kidney disease (DKD) beyond proteinuria is increasing. Metabolomics is a popular method to identify mechanisms and biomarkers. We investigated urinary targeted metabolomics in DKD patients.

Methods: We conducted a targeted metabolomics study of 26 urinary metabolites in consecutive patients with DKD stage 1 to 5 (n = 208) and healthy controls (n = 26). The relationships between estimated glomerular filtration rate (eGFR) or urine protein-creatinine ratio (UPCR) and metabolites were evaluated. Multivariate Cox analysis was used to estimate relationships between urinary metabolites and the target outcome, end-stage renal disease (ESRD). C statistics and time-dependent receiver operating characteristics (ROC) were used to assess diagnostic validity.

Results: During a median 4.5 years of follow-up, 103 patients (44.0%) progressed to ESRD and 65 (27.8%) died. The median fold changes of nine metabolites belonged to monosaccharide and tricarboxylic acid (TCA) cycle metabolites tended to increase with DKD stage. Myo-inositol, choline, and citrates were correlated with eGFR and choline, while mannose and myo-inositol were correlated with UPCR. Elevated urinary monosaccharide and TCA cycle metabolites showed associations with increased morality and ESRD progression. The predictive power of ESRD progression was high, in the order of choline, myo-inositol, and citrate. Although urinary metabolites alone were less predictive than serum creatinine or UPCR, myo-inositol had additive effect with serum creatinine and UPCR. In time-dependent ROC, myo-inositol was more predictive than UPCR of 1-year ESRD progression prediction.

Conclusion: Myo-inositol can be used as an additive biomarker of ESRD progression in DKD.

Keywords: Diabetic nephropathies, End-stage renal disease, Metabolomics, Myo-inositol

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Introduction

The current gold standards of measuring renal function are urinary clearance of insulin or ^{125}I-iothalamate. However, due to difficulty and inconvenience of assessment many alternative biomarkers have been devised, especially endogenously produced biomarkers [1,2]. Estimated glomerular filtration rate (eGFR) calculated by serum creatinine and cystatin C concentrations show excellent correlations with gold standards and are used in routine practice to evaluate renal function. Proteinuria measurement is commonly used together with eGFR as predictive biomarkers in chronic kidney disease (CKD) [3]. However, current renal function estimation tools have some measurement limitations. They can be biologically confounded by muscle mass, thyroid function, and liver function [4,5]. There are also blind-spot patients whose renal function deteriorates faster than expected. Interest in developing new biomarkers to supplement or overcome the limitations of current biomarkers remains high.

Metabolomics is in the spotlight to identify new disease-specific biomarkers [6–8] and is amenable to application to many types of samples such as blood, tissue, and secretions [9]. Urine metabolomics, which includes analyses of metabolic break-down products and reveals renal condition by examining biological waste made by the kidneys, is commonly used in renal diseases [10,11]. As mechanisms depend on the primary cause of kidney disease, disease-specific urinary metabolomics are usually performed [12,13].

Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease (ESRD) and is expected to increase in prevalence [14]. Therefore, several urinary metabolomics methods have been developed for DKD. Previous metabolomics studies reported changes in mitochondrial and fatty acid metabolites in DKD patients [15–19], but the results vary.

To investigate metabolomics changes in early and late-stage DKD, we previously performed untargeted serum and urine metabolomics in *db/m* and *db/db* mice. We observed early phase increase of BCAA and homocysteine-methionine metabolism and late phase increase of ketone and fatty acid metabolism [16]. In the present study, we aimed to assess changes of urinary metabolites identified in the previous study according to DKD stages and evaluate the ability of urinary metabolites to predict progression to ESRD.

Methods

This study was conducted under the approval of the Research Ethics Committee of the Seoul National University Boramae Medical Center (SMG-BMC, No. 30-2019-1). All procedures followed the ethical standards of the institutional research committee and the 2013 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from participants before the collection of urine.

Study design and population

From June 2011 to June 2018, we prospectively enrolled consecutive patients with DKD stages 1 to 5 (n = 208) and healthy controls (n = 26) with normal kidney function and without diabetes at SMG-BMC. DKD was defined as patients who were diagnosed with diabetes before enrollment, presence of proteinuria (≥either urine dipstick albumin 1+, ≥300 mg/g urine albumin-creatinine ratio (UACR), or ≥0.15 g/g urine protein-creatinine ratio [UPCR]), and absence of glomerulonephritis.

DKD stages were divided based on the CKD Epidemiology Collaboration equation (CKD-EPI) eGFR using serum creatinine [20]. To be eligible for the study, a patient had to be older than 18 years and not on dialysis at the time of enrollment. Urine samples were collected at the time of enrollment.

Targeted metabolites selection

In our results from untargeted metabolomic studies in mice [16], urinary carbohydrates (glucose, mannose, xylose) and tricarboxylic acid (TCA) cycle intermediates (lactate, pyruvate, 2-oxoglutarate, citrate, fumarate, and 3-metyl-2oxovalerate) showed significant differences at both 8 and 20 weeks between *db/m* and *db/db* mice. Urinary amino acids (isoleucine, leucine, valine, creatine, taurine, 2-oxoisocaproate, methionine, *N*,*N*-dimethylglycine [DMG], and sarcosine) were elevated at 8 weeks and ketogenesis metabolites (3-hydroxybutyrate, acetoacetate, O-acetylcarnitine) were elevated at 20 weeks from *db/db*

to *db/m* mice. Since some urinary metabolites (glucarate, 3-metyl-2oxovalerate, methionine, sarcosine, 3-hydroxybutyrate, and acetoacetate) that showed significant differences in a previous animal study could not be measured in the human sample used in the present study, we measured related metabolites (pyroglutamate, trimethylamine *N*-oxide [TMNO], betaine, threonine, and carnitine) instead. Although we did not observe myo-inositol in the previous animal study, based on untargeted metabolomics results from a study of streptozotocin-induced diabetic mice revealing elevated urinary myo-inositol levels [21] and known predictive power in DKD [22,23], we included myo-inositol among the targeted metabolomics. Finally, 28 urinary metabolites were analyzed (Supplementary Table 1, available online).

Sample preparation for metabolite analysis

Prior to nuclear magnetic resonance (NMR) experiments, frozen urine samples stored at –80 ºC were thawed at room temperature. Urine samples were filtered through Amicon Ultra centrifugal filters for 500 μL – 3K (Millipore) at 17,870 ×*g* for 10 minutes at 4 °C to remove protein. The resulting 300-μL supernatant from the urine sample was mixed with 300 μL of 0.2-M sodium phosphate buffer (pH 7.0) and 1-mM sodium azide in deuterium oxide (D_2O). After adjusting the pH to 7.0 ± 0.1, 540 μL of sample was mixed with 60 μL of 5-mM 3-(trimethylsilyl) propionic 2,2,3,3-acid (TSP) in D_2O, and the 600-μL samples were placed in 5-mm Bruker SampleJet NMR tubes (Z112273; Bruker BioSpin AG).

^{1}H nuclear magnetic resonance experiments

One-dimensional (1D) ^{1}H NMR spectra were acquired with an Ascend 800-MHz AVANCE III HD Bruker spectrometer using a triple-resonance 5-mm CPTIC cryogenic probe (Bruker BioSpin AG). To acquire 1D ^{1}H spectra of the urine samples, Bruker standard 1D nuclear Overhauser enhancement spectroscopy (NOESY)-presat (noesypr1d) pulse sequences were used as follows: *RD* – 90° – short delay – 90° – mixing – 90° – *Acq*, with relaxation delay (*RD*) = 4.0 seconds, short delay = 12.18 μs, n = 128, dummy scans = 16, acquisition time (*Acq*) = 2.0 seconds, and mixing time (mixing) = 10 milliseconds. The water signal was suppressed at the water peak during the *RD* and mixing time. Fourier domain points were acquired at 65,536 data points with a spectral width of 20 ppm.

The NMR data were processed using TopSpin (ver. 3.1; Bruker BioSpin). All spectra were manually baseline-corrected and phase-corrected. The processed NMR spectra were imported into Chenomx for identification and quantification, and the 800-MHz Chenomx library (ver. 7.1; Chenomx) was used to identify individual compounds. The assignment of ambiguous peaks due to peak overlap was confirmed by spiking with standard compounds. Signal assignments for representative samples were facilitated by the acquisition of two-dimensional correlation spectroscopy and heteronuclear single quantum correlation. The quantification of urinary metabolites was achieved using Chenomx, which used the concentrations of TSP to determine the concentrations of individual compounds. The urinary concentrations were normalized to the levels of creatinine (metabolite μM/creatinine mM).

Clinical data collection

Baseline clinical parameters such as age, sex, body mass index, comorbidities, and laboratory findings, including serum level of complete blood cell counts, aspartate aminotransferase, alanine aminotransferase, albumin, creatinine, cholesterol, uric acid, fasting glucose, hemoglobin A1c (HbA1c), and UPCR were collected from electronic medical records.

Study outcomes

The primary study outcome was the number of ESRD events (maintenance dialysis or kidney transplantation). ESRD was defined as hemodialysis more than 90 days, surgery for peritoneal dialysis or kidney plantation. Information regarding ESRD was obtained from the Korean Society of Nephrology database [24]. All-cause mortality data were obtained from the National Database of Statistics Korea. Patients were followed up until their deaths or August 2018, whichever came first. The composite outcome was defined as the occurrence of either ESRD or all-cause mortality.

Statistical analysis

Since metabolite concentrations for each value were not normally distributed, they were analyzed after natural logarithmic transformation (Ln [urinary metabolites/creatinine]) (Supplementary Fig. 1 and 2, available online). We conducted cross-sectional and longitudinal analyses. Cross-sectional analysis was used to compare metabolic differences among healthy control and DKD groups. We expressed categorical variables as percentages for all patients and continuous variables that follow the normal distribution curve as means ± standard deviations. The trends according to DKD stage were compared using linear regression for continuous variables and the Cochran-Armitage trend test for categorical variables. Mann-Whitney *post hoc* analysis was applied. Correlations between metabolites and eGFR or UPCR were determined using Pearson correlation analysis.

Survival analysis was conducted to investigate relationships between urinary metabolites and outcome. Kaplan-Meier survival analysis and the log-rank method were used to compare outcomes among metabolic quantiles. Multivariate Cox regression adjusted for the effects of traditional risk factors was performed by backward stepwise model selection. The area under the receiver operating characteristic (ROC) curve (AUC) was used in both simple and time-dependent manner [25]. All tests were two-tailed and $p < 0.05$ was considered indicative of statistical significance. The p-value of <0.1 was used exceptionally when selecting variables in Cox analysis by backward stepwise method. Statistical analyses were performed using IBM SPSS version 25.0 (IBM Corp.) and R version 3.5.1 (The Comprehensive R Archive Network; http://cran.r-project.org).

Results

Evaluations of baseline characteristics according to diabetic kidney disease stages

The median follow-up duration was 54.0 months (interquartile range [IQR], 25.0–79.0 months). Healthy controls and DKD stage 1–2 patients were younger than patients with stage 3 or higher DKD (control: 35.8 ± 15.2 years, DKD stage 1–2: 49.3 ± 17.8 years, and DKD stage 3 over: 63.0±12.2 years; p for trend < 0.001). The advanced DKD patient groups had higher prevalence of hypertension (p for trend < 0.001). There was significant difference in serum creatinine between control group and early stage DKD (stages 1–2) (control: 0.8 ± 0.1 mg/dL and DKD stage 1–2: 0.9 ± 0.2 mg/dL; p = 0.003). Moreover, the differences were more prominent in eGFR (control: 112.5 ± 11.8 mL/min/1.73 m^2 and DKD stage 1–2: 91.0 ± 19.3 mL/min/1.73 m^2; p < 0.001) and proteinuria (median [IQR] of UPCR: control, 0.1 [0.0–0.1] g/g and DKD stage 1–2, 0.7 [0.3– 5.5]; p < 0.001). The more advanced the DKD stages, the more proteinuria was observed (p for trend < 0.001) (Table 1).

There were no significant trends in glycemic control according to DKD stages in either fasting glucose level or HbA1c (fasting glucose: p for trend = 0.28; HbA1c: p for trend = 0.20) (Table 1). When we divided DKD patients into two groups by eGFR 30 mL/min/1.73 m^2, there was also no difference in fasting glucose level between groups (fasting glucose: DKD stage 1–3, 134.0 ± 48.2 mg/dL; DKD stage 4–5, 137.3 ± 59.8 mg/dL; p = 0.68). However, the advanced DKD groups with eGFR of ≤30 mL/min/1.73 m^2 tended to have better glycemic control (HbA1c: DKD stage 1–3, 7.3% ± 1.2%; DKD stage 4–5, 6.9% ± 1.3%; p = 0.02).

Evaluations of relationships between urinary metabolite trends and diabetic kidney disease stages

The median fold change of each metabolite (Ln [urinary metabolites/creatinine]) in DKD stage groups were compared with controls. There were no significant tendencies for ketogenesis metabolites according to DKD stages (acetone: p for trend = 0.06, O-acetylcarnitine: p for trend = 0.24) (Table 2). A total of 19 metabolites showed significant trends across DKD stages, and 10 urinary metabolites (glucose, mannose, xylose, myo-inositol, glycerol, lactate, citrate, fumarate, creatine, and choline) maintained significance in the *post hoc* analysis (Table 2; Supplementary Fig. 3, available online).

Myo-inositol ($R^2 = 0.442$, $p < 0.001$), choline ($R^2 = 0.293$, $p < 0.001$) and citrate ($R^2 = 0.175$, $p < 0.001$) were correlated with eGFR and choline ($R^2 = 0.281$, $p < 0.001$), mannose ($R^2 = 0.260$, $p < 0.001$) and myo-inositol ($R^2 = 0.236$, $p < 0.001$) were correlated with UPCR. The urinary metabolites were more strongly correlated with eGFR than with UPCR (Fig. 1; Supplementary Fig. 4, available online).

Table 1. Baseline characteristics of DKD patients and healthy controls

Characteristic	Total	Control	DKD stage					p for trend
			1–2	3A	3B	4	5	
No. of patient	234	26	32	20	37	61	58	
Age (yr)	58.5 ± 16.3	35.8 ± 15.2	49.3 ± 17.8	59.5 ± 10.3	65.5 ± 11.3	65.1 ± 11.8	60.9 ± 13.1	<0.001
Male sex	147 (62.8)	15 (57.7)	21 (65.6)	17 (85.0)	23 (62.2)	36 (59.0)	35 (60.3)	0.74
BMI (kg/m^2)	23.6 ± 3.8	24.0 ± 3.4	25.6 ± 3.6	23.5 ± 4.0	23.7 ± 3.7	23.5 ± 3.4	22.8 ± 4.3	0.14
Hypertension	150 (64.1)	4 (15.4)	16 (50.0)	13 (65.0)	28 (75.7)	45 (73.8)	44 (75.9)	<0.001
Laboratory findings								
Creatinine (mg/dL)	2.8 ± 2.2	0.8 ± 0.1	0.9 ± 0.2	1.5 ± 0.2	1.8 ± 0.3	2.7 ± 0.6	5.8 ± 2.1	<0.001
eGFR (mL/min/1.73 m^2)	43.0 ± 36.6	112.5 ± 11.8	91.0 ± 19.3	50.1 ± 3.6	36.2 ± 4.4	21.6 ± 4.3	9.6 ± 2.7	<0.001
Hemoglobin (g/dL)	11.6 ± 2.2	14.4 ± 1.8	13.2 ± 2.5	12.6 ± 1.8	11.5 ± 1.6	11.0 ± 1.4	9.8 ± 1.2	<0.001
Calcium (mg/dL)	8.6 ± 0.8	9.0 ± 0.9	8.9 ± 0.7	9.1 ± 0.4	8.7 ± 0.6	8.7 ± 0.6	7.9 ± 0.9	<0.001
Phosphorus (mg/dL)	4.0 ± 0.8	3.5 ± 0.6	3.7 ± 0.6	3.8 ± 0.6	3.8 ± 0.6	3.9 ± 0.8	4.8 ± 0.9	<0.001
Albumin (g/dL)	3.8 ± 0.6	4.3 ± 0.4	4.0 ± 0.6	4.0 ± 0.3	3.8 ± 0.5	3.8 ± 0.5	3.5 ± 0.4	<0.001
AST (IU/L)	21.5 ± 10.3	25.1 ± 14.9	23.7 ± 10.3	19.6 ± 11.5	24.0 ± 11.2	20.3 ± 6.8	18.8 ± 8.9	0.038
ALT (IU/L)	20.1 ± 14.7	28.9 ± 30.5	24.0 ± 13.1	16.2 ± 7.1	21.3 ± 13.0	17.4 ± 8.9	16.8 ± 8.5	0.003
Fasting glucose (mg/dL)	132.8 ± 52.8	109.5 ± 19.4	125.9 ± 30.4	135.2 ± 53.5	140.6 ± 57.3	135.4 ± 43.1	139.1 ± 72.6	0.28
HbA1c (%)	7.0 ± 1.3	6.0 ± 0.4	7.3 ± 1.4	7.2 ± 1.5	7.3 ± 1.0	6.9 ± 1.2	6.8 ± 1.4	0.20
Uric acid (mg/dL)	7.0 ± 2.1	5.0 ± 1.6	6.0 ± 1.9	7.1 ± 2.2	6.9 ± 2.2	7.8 ± 2.0	7.8 ± 1.6	<0.001
TC (mg/dL)	158.5 (136–182)	175.5 (140–186)	161 (138–190)	173 (148–202)	157 (118–171)	152 (136–176)	154 (129–182.5)	0.15
Triglyceride (mg/dL)	124 (92–199)	154 (104–194)	114 (94.5–150.5)	113 (96–128.5)	135 (101–256)	121 (93–186)	133 (81–212)	0.95
HDL (mg/dL)	41 (33.5–50.5)	46.0 (41–56)	43 (36.5–54.5)	42 (38.5–46.5)	41 (32–49)	44 (36–51)	34 (29–38)	0.001
LDL (mg/dL)	86 (67–107)	90.5 (86.0–116.5)	86 (62–106)	99 (74.5–132.5)	79.5 (58–90)	75.5 (66–101)	89.5 (72.5–117)	0.16
UPCR (g/g)	2.0 (0.4–5.7)	0.1 (0.0–0.1)	0.7 (0.3–5.5)	1.7 (0.2–5.7)	0.9 (0.3–6.3)	1.9 (0.6–3.5)	5.3 (3.2–8.7)	<0.001
Metformin usage	48 (20.5)	0 (0)	8 (57.1)	11 (61.1)	13 (65.0)	4 (6.6)	4 (6.9)	<0.001

Data are expressed as number only, mean ± standard deviation, number (%), median (range), or interquartile range.
ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate (by CKD-EPI creatinine equation); HbA1C, hemoglobin A1c; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; UPCR, urine protein-creatinine ratio.

Table 2. Median fold changes of urine metabolites according to DKD stage, compared with the control group

Ln (metabolites/Cr)[a]	Stage 1–2/control	Stage 3A/control	Stage 3B/control	Stage 4/control	Stage 5/control	p for trend
Glucose	1.066***	1.079***	1.338**	1.511*	3.206***	<0.001
Mannose	0.743***	0.855**	1.032***	1.059***	2.143***	<0.001
Xylose	0.330*	−0.129	−0.092	−0.430**	−0.458***	<0.001
Myo-inositol	0.782**	1.240***	1.943***	2.254***	2.811***	<0.001
Glycerol	0.270	0.551	0.713**	0.594**	0.718***	<0.001
Lactate	0.253*	0.311	0.579**	0.778***	1.439***	<0.001
Pyruvate	0.075	−0.274	−0.407	−0.287	−0.091	0.57
Citrate	0.325	−0.461*	−0.417*	−1.524***	−1.097***	<0.001
2-Oxoglutarate	0.519	−0.224	−0.038	−0.066	0.353	0.83
Succinate	0.613***	0.768**	1.467***	0.707**	0.789***	0.002
Fumarate	0.954**	0.749**	1.096***	1.164***	1.650***	<0.001
Pyroglutamate	0.144	−0.023	0.222	0.208	0.494***	<0.001
Acetone	0.414	0.559	0.498*	0.598*	0.609**	0.06
O-Acetylcarnitine	−0.735	−0.187	−0.469	−0.438	−0.317	0.24
Isoleucine	0.010	0.094	−0.078	−0.306	0.721**	0.003
Leucine	−0.052	−0.044	−0.022	0.037	0.662***	<0.001
Valine	−0.032	−0.108	−0.074	−0.151	0.718***	0.001
Creatine	−0.707	−0.917*	−1.135***	−1.334***	−1.195***	<0.001
Taurine	−0.291	−0.428	−0.279	−0.315*	−0.606***	0.005
Threonine	0.008	−0.128	−0.281	−0.218	0.555	0.28
Carnitine	−0.626	0.059	−0.719	−0.394	−0.536	0.27
2-Oxoisocaproate	0.047	−0.205	−0.201	0.027	0.085	0.28
Choline	0.559*	0.580*	1.064***	1.651***	2.405***	<0.001
DMG	0.412*	0.175	0.124	−0.063	−0.175	<0.001
TMAO	0.411	0.595	0.288	0.652	1.098**	0.002
Betaine	0.768**	1.199***	0.843***	0.865***	0.679***	0.02

Cr, creatinine; DKD, diabetic kidney disease; DMG, *N,N*-dimethylglycine; TMNO, trimethylamine *N*-oxide.
[a]All metabolites were normalized to the levels of urinary creatinine concentration measured by nuclear magnetic resonance and natural logarithmic transformed (Ln [urinary metabolites/creatinine, μM/creatinine mM]).
Post hoc analysis with Mann-Whitney test; *p < 0.05, **p < 0.01, ***p < 0.001 (compared each DKD groups with control).

Metabolites associated with end-stage renal disease progression

During the follow-up period, 103 participants (44.0%) progressed to ESRD and no patients in the control group progressed to ESRD. In Kaplan-Meier analysis, 17 metabolites were associated with ESRD progression (Fig. 2; Supplementary Fig. 5, available online). Urinary monosaccharide concentration was closely related to ESRD progression. Increased urinary glucose, mannose, and myo-inositol level were associated with greater progression to ESRD. On the contrary, decreased urinary xylose concentration was associated with ESRD progression. The concentrations of urinary metabolites associated with ketogenesis (acetone and acetylcarnitine) were not associated with ESRD progression. Urinary metabolites were related to ESRD progression associated with the TCA cycle, amino acid, and choline pathway. An increase in most urine metabolite concentrations (glycerol, lactate, fumarate, pyroglutamate, isoleucine, leucine, valine, threonine, choline, and TMNO) and a decrease in some metabolite concentrations (citrate, taurine, and DMG) were associated with increase in ESRD progression.

After adjustments for baseline characteristics (age, sex, eGFR, UPCR, HbA1c, and other laboratory findings) by multivariate Cox analysis, 12 metabolites were still associated with ESRD progression (Table 3). All metabolites with decreased urinary concentrations associated with

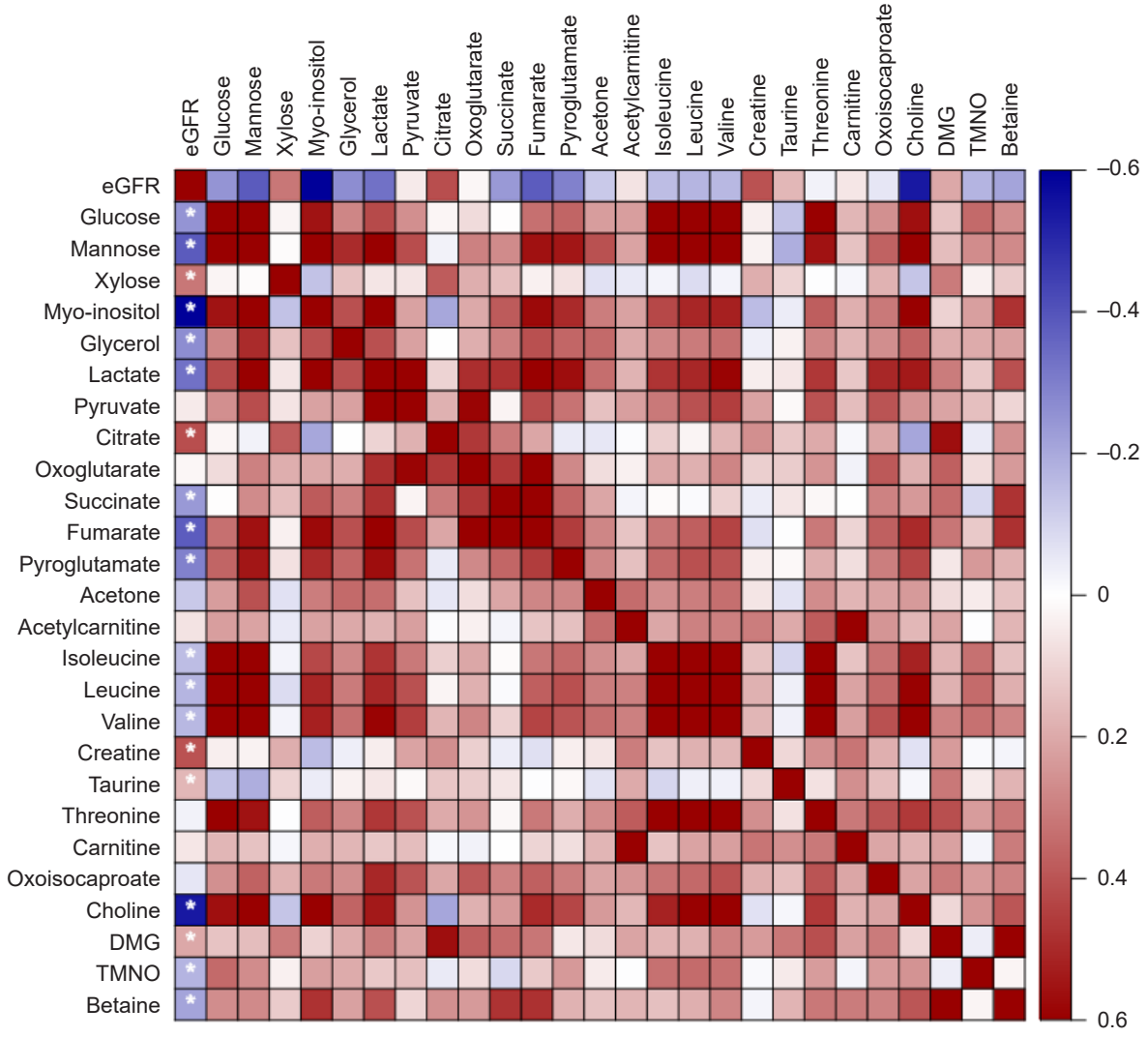

Figure 1. Correlation matrix of urine metabolite concentrations and eGFR. Correlations among targeted natural log urine metabolites per urine creatinine (Ln [metabolites/creatinine]) and eGFR were obtained by Pearson correlation coefficient for each pair of metabolites. The color scheme corresponds to correlation strength as shown by the color bar.
DMG, *N,N*-dimethylglycine; eGFR, estimated glomerular filtration rate; TMNO, trimethylamine *N*-oxide.

ESRD progression in Kaplan-Meier were not significant in Cox analysis. Increased concentrations of nine urinary metabolites (glucose, mannose, myo-inositol, glycerol, lactate, fumarate, pyroglutamtae, leucine, and valine) were associated with increased risk of ESRD progression in both Kaplan-Meyer and Cox analysis.

Metabolites associated with all-cause mortality and composite outcome

Sixty-five patients (27.8%) died, and the composite outcome was achieved in 135 participants (57.7%). There were no mortalities in the control group. In Kaplan-Meier analysis, increased urinary concentrations of 14 metabolites were associated with all-cause mortality (Supplementary Fig. 6, available online) (glucose, mannose, myo-inositol, glycerol, lactate, fumarate, pyroglutamate, isoleucine, leucine, valine threonine, choline, TMNO, and betaine) and 16 metabolites were associated with the composite outcome (Supplementary Fig. 7, available online). Without betaine, 13 urinary metabolites that increased the risk of all-cause mortality were also associated with increased risk of ESRD

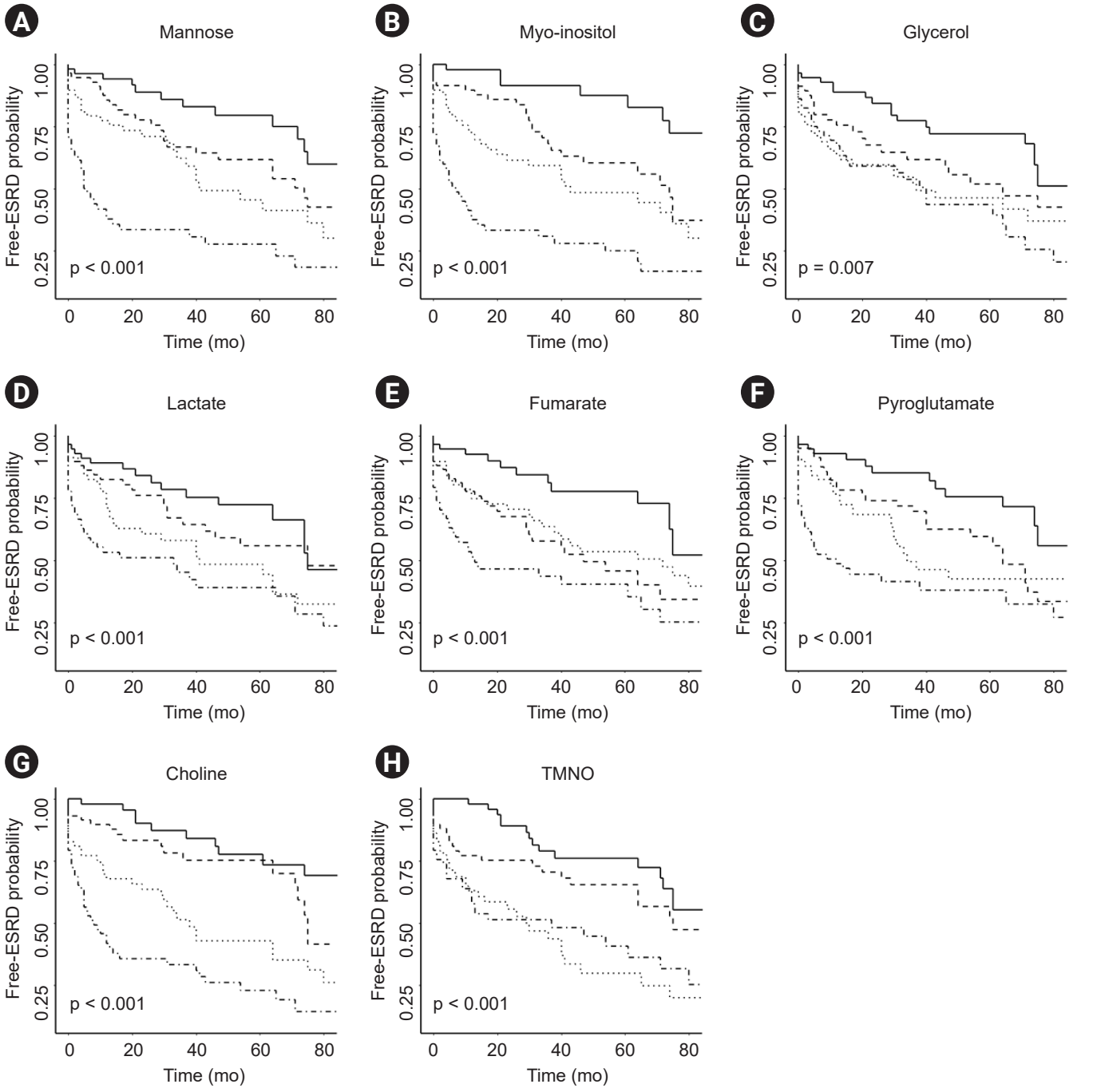

Figure 2. Kaplan-Meier survival curves of selected metabolites for ESRD progression. Patients with first (solid), second (dashed), third (dotted) and forth (dot dash) quantiles of each level of natural log urine metabolites per urine creatinine (Ln [metabolites/creatinine]) were subjected to these analyses. (A) Mannose. (B) Myo-inositol. (C) Clycerol. (D) Lactate. (E) Fumarate. (F) Pyroglutamate. (G) Choline. (H) Trimethylamine *N*-oxide (TMNO).
ESRD, end-stage renal disease.

Table 3. Associations of urinary metabolites with renal outcomes, using a backward stepwise multivariate Cox model

Ln (metabolites/Cr)[d]	Unadjusted			Model 1[a]			Model 2[b]			Model 3[c]		
	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Glucose	1.35	1.22–1.48	<0.001	1.23	1.11–1.36	<0.001	1.30	1.16–1.46	<0.001	1.22	1.08–1.38	0.001
Mannose	1.69	1.45–1.97	<0.001	1.45	1.23–1.71	<0.001	1.44	1.19–1.73	<0.001	1.34	1.09–1.65	0.006
Xylose	0.50	0.37–0.69	<0.001									
Myo-inositol	2.13	1.71–2.65	<0.001	1.45	1.12–1.88	0.005	1.54	1.16–2.05	0.003	1.44	1.06–1.95	0.02
Glycerol	1.49	1.19–1.87	0.001	1.52	1.19–1.95	0.001	1.45	1.12–1.87	0.005	1.52	1.12–2.05	0.007
Lactate	1.41	1.20–1.65	<0.001	1.43	1.17–1.76	0.001	1.45	1.19–1.78	<0.001	1.33	1.07–1.64	0.01
Pyruvate	1.15	0.95–1.39	0.14	1.52	1.23–1.88	<0.001	1.54	1.23–1.94	<0.001	1.34	1.07–1.67	0.01
Citrate	0.67	0.58–0.78	<0.001									
Oxoglutarate	1.04	0.85–1.27	0.71	1.33	1.07–1.66	0.01	1.51	1.19–1.91	0.001	1.33	1.05–1.68	0.02
Succinate	1.06	0.87–1.30	0.56									
Fumarate	1.40	1.17–1.67	<0.001	1.37	1.10–1.72	0.005	1.45	1.15–1.83	0.002	1.41	1.12–1.78	0.004
Pyroglutamate	3.98	2.52–6.29	<0.001	2.82	1.72–4.60	<0.001	3.85	2.26–6.54	<0.001	3.23	1.71–6.11	<0.001
Acetone	1.13	0.92–1.39	0.25									
Acetylcarnitine	1.08	0.88–1.33	0.45	1.36	1.08–1.73	0.01	1.37	1.04–1.80	0.03			
Isoleucine	1.89	1.55–2.30	<0.001	1.49	1.23–1.81	<0.001	1.47	1.19–1.82	<0.001	1.23	0.99–1.53	0.07
Leucine	2.23	1.77–2.82	<0.001	1.63	1.30–2.05	<0.001	1.62	1.26–2.07	<0.001	1.43	1.09–1.87	0.009
Valine	1.78	1.45–2.17	<0.001	1.42	1.17–1.72	<0.001	1.57	1.29–1.89	<0.001	1.28	1.02–1.60	0.03
Creatine	0.79	0.63–0.997	0.047	1.32	0.999–1.75	0.05						
Taurine	0.74	0.61–0.90	0.002									
Threonine	1.38	1.15–1.65	0.001	1.24	1.04–1.48	0.02	1.24	1.00–1.54	0.049			
Carnitine	1.03	0.87–1.23	0.71	1.24	1.01–1.51	0.04						
Oxoisocaproate	1.60	1.13–2.26	0.008	2.67	1.80–3.97	<0.001	2.63	1.73–4.01	<0.001	2.06	1.34–3.18	0.001
Choline	1.78	1.53–2.06	<0.001	1.33	1.10–1.61	0.004	1.24	0.999–1.53	0.05	1.21	0.98–1.50	0.07
DMG	0.60	0.45–0.81	0.001									
TMNO	1.32	1.10–1.58	0.003	1.21	1.01–1.44	0.04	1.28	1.04–1.56	0.02			
Betaine	0.95	0.74–1.21	0.66									

Cr, creatinine; CI, confidence interval; DMG, *N,N*-dimethylglycine; HR, hazard ratio; TMNO, trimethylamine *N*-oxide.
[a]Adjusted for age, sex, hypertension, and estimated glomerular filtration rate. [b]Adjusted for model 1 variables, plus hemoglobin, albumin, aspartate transaminase, alanine transaminase, cholesterol, and uric acid. [c]Adjusted for model 2 variables, plus spot urine protein-creatinine ratio and hemoglobin A1c. [d]All metabolites were normalized to the levels of urinary creatinine concentration measured by nuclear magnetic resonance and natural logarithmic transformed (Ln [urinary metabolites/creatinine, μM/creatinine mM]).

and composite outcome. Reductions of three urinary metabolites (xylose, citrate, and creatine) were associated with increased risk of composite outcome, of which creatine was not significant in ESRD progression and all-cause mortality but was only associated with the composite outcome.

In multivariate Cox analysis, 11 metabolites were associated with all-cause mortality (Supplementary Table 2, available online), and 14 metabolites were associated with the composite outcome (Supplementary Table 3, available online). The increased concentrations of three urinary monosaccharides (glucose, mannose, and myo-inositol) were associated with increased risk of both ESRD progression and all-cause mortality. However, urinary ketogenesis metabolites were associated with neither outcome. TCA cycle metabolites were less associated with risk of all-cause mortality than ESRD progression. Decreases in urine concentrations of choline and threonine were only associated with death.

Receiver operating characteristic analysis

We compared serum creatinine concentration and UPCR with each urinary metabolite by ROC analysis to determine predictive utility for progression to ESRD. Choline, myo-inositol, and citrate were the most predictive urine metabolites, although they were not superior to serum creatinine and UPCR alone (Supplementary Table 4, available online). To assess additive effects of each urinary metabolite to serum creatinine concentration and UPCR in prediction of ESRD progression, the net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were used (Table 4). Only myo-inositol improved prediction (NRI, 2.9%, p = 0.03; IDI, 35.1%, p = 0.02).

For the three most predictive urine metabolites, the predictive power of ESRD progression at the first, second, and fourth years from the time of sample collection was evaluated by time-dependent ROC. In time-dependent ROC analysis, serum creatinine was the best biomarker for the shorter term, and UPCR was the best for the longer term. Choline and myo-inositol were more predictive than UPCR for 12-month ESRD progression prediction (Fig. 3; Supplementary Table 5, available online).

Discussion

In this large prospective Korean DKD cohort, we performed targeted NMR-based urinary metabolomics including 26 metabolites in consecutive stages of DKD patients and performed various analyses to reveal urine metabolites associated with ESRD progression. When comparing urinary metabolites according to DKD stages, monosaccharide, some TCA cycle pathway intermediates, creatine, and choline showed significant trends. Among them, there were correlations with eGFR in the order of myo-inositol, choline, and citrate, and with UPCR in the order of choline, mannose, and myo-inositol. In survival analysis, increased concentrations of nine urinary metabolites (glucose, mannose, myo-inositol, glycerol, lactate, fumarate, pyroglutamtae, leucine, and valine) predicted ESRD progression. Of these, urinary myo-inositol had an additive effect on serum creatinine and UPCR in predicting ESRD progression. Based on our multidisciplinary analysis, urinary myo-inositol concentration can increase predictive power when used in combination with serum creatinine and UPCR in ESRD progression.

In the present study, we validated our previous results

Table 4. Additive effects of urinary metabolites to predict end-stage renal disease progression, analyzed by net reclassification improvement (NRI) and integrated discrimination improvement (IDI)

Urinary metabolite	AUC (95% CI)	DeLong test	NRI		IDI	
			p-value	95% CI	p-value	95% CI
Creatinine + UPCR	0.905 (0.865–0.945)	Reference	Reference		Reference	
Creatinine + UPCR + choline	0.904 (0.864–0.945)	0.437	0.32	1.2 (–1.6 to 4.7)	0.23	24.6 (–67.8 to 54.7)
Creatinine + UPCR + myo-inositol	0.904 (0.864–0.945)	0.430	0.03	2.9 (0.1 to 8.8)	0.02	35.1 (5.0 to 51.2)
Creatinine + UPCR + citrate	0.911 (0.873–0.949)	0.436	0.63	0.1 (–1.3 to 2.7)	0.16	10.0 (–11.3 to 25.2)
Creatinine +UPCR+ 3 metabolite (choline + myo-inositol + citrate)	0.888 (0.847–9.300)	0.264	0.03	3.6 (0.0 to 9.2)	0.02	25.2 (3.4 to 44.1)

AUC, area under the receiver operating characteristic curve; CI, confidence interval; UPCR, urine protein-creatinine ratio.

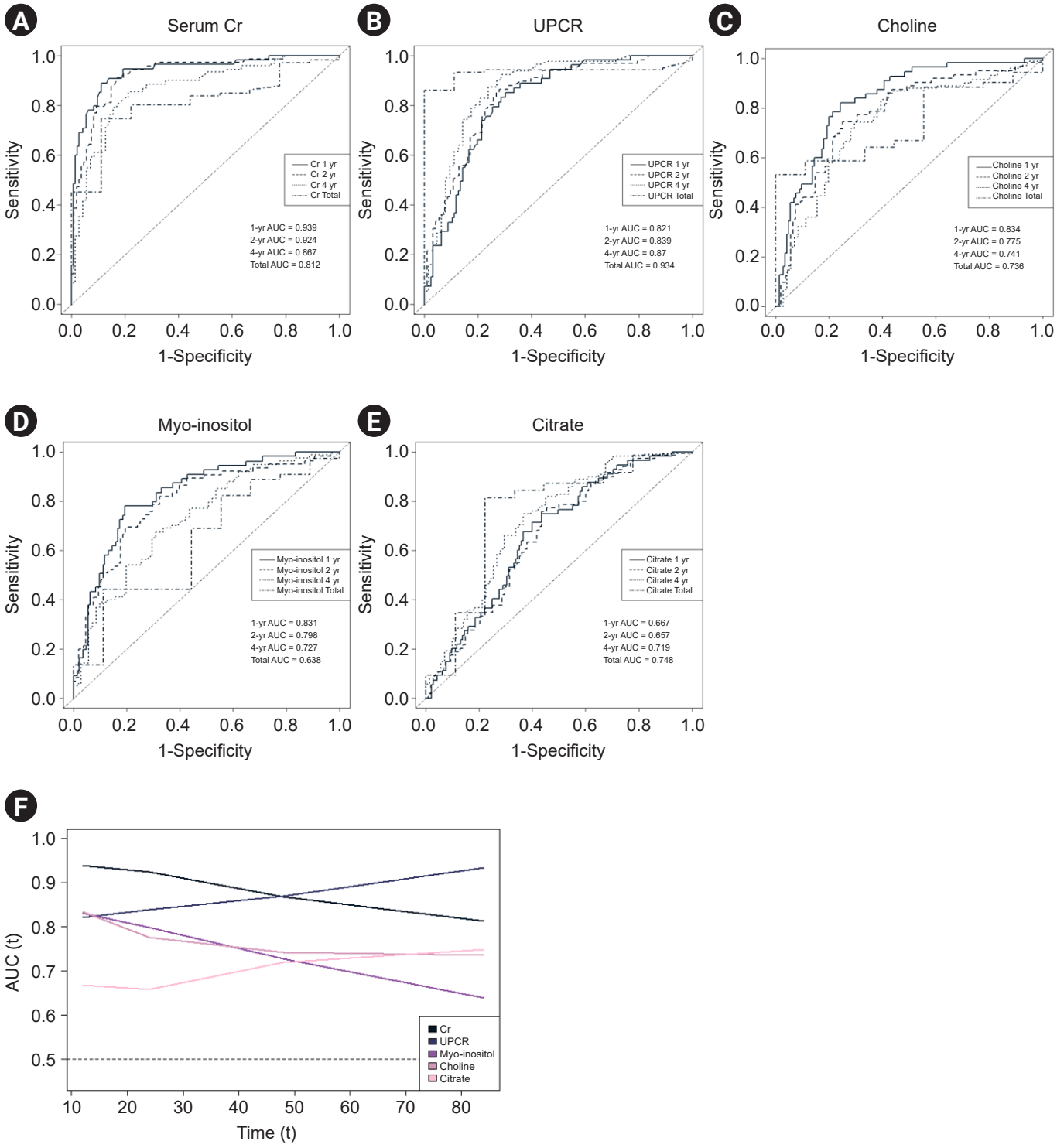

Figure 3. Time-dependent ROC curves for predicting ESRD progression. Time-dependent ROC curves for 1, 2 and 4 years after sampling compared with total follow-up period of ESRD progression. (A) Serum creatinine (Cr), (B) urine protein-creatinine ratio (UPCR), (C) choline, (D) myo-inositol, and (E) citrate. The three most predictive metabolites were compared with serum Cr and UPCR. Metabolites were used as natural log urine metabolites per urine creatinine. (F) Areas under the ROC curve (AUCs) of aforementioned variables at each time after sample acquisition with ESRD progression as a status variable.
ESRD, end-stage renal disease; ROC, receiver operating characteristics.

based on untargeted metabolomics between *db/db* and *db/m* mice by performing urine targeted metabolomics for human DKD patients at various stages of disease and healthy controls [16]. In accordance with the results of our animal study, urine monosaccharide (glucose and mannose), TCA cycle metabolites (lactate, pyruvate, and fumarate), and some amino acids (creatine and taurine) decreased in both early and advanced DKD patients. Contrary to our urinary metabolomics study of mice, in which we found that xylose and citrate increased in both early and late phases, the concentrations of both substances decreased in patients with DKD stage 3A or higher. Urinary branched-chain amino acid (BCAA; leucine and valine) was elevated in early phases of the mouse model but were decreased in DKD stage 1–3B and increased in DKD stage 5 in human samples. The ketogenic pathway metabolites (acetone and O-acetylcarnitine) were also significantly elevated in late phase in the murine model, but there were no significant changes according to human DKD groups and urinary acetone concentrations were elevated in DKD patients compared to controls.

Due to innovative development, omic studies are widely used to identify biomarkers and mechanisms [26]. Metabolomics, focusing on final substances that closely reflect phenotypes, has recently been applied to CKD to identify underlying causes [6,27]. As DKD is a leading cause of ESRD, many human and animal metabolomics studies have been performed, and have reported that mitochondrial function is deregulated and bioenergy metabolism is reduced in DKD [6,17,28–30]. However, few studies have performed urine metabolomics and estimated long-term predictive efficacy in DKD patients. A previous gas chromatography-mass spectrometry-based study targeted urine metabolomics (urinary metabolites, n = 94), comparing diabetic patients with and without CKD, and showed significant alterations of several metabolites (13 metabolites including citrate, BCAA catabolism-related metabolites). These results suggested global suppuration of mitochondrial activity in DKD by pathway and network analysis [28]. Moreover, targeted NMR metabolomics of early type 1 diabetes patients from the Finnish Diabetic Nephropathy (FinnDiane) study reported that elevated BCAA (isoleucine, leucine, and valine) and other urinary metabolites (pseudouridine and threonine) and decreased urinary citrate are associated with worse albuminuria categories or progression to ESRD [31]. Considering that increased urinary BCAA was also associated with ESRD progression in our study, and that participants in the FinnDiane study had better renal function than our patients (eGFR [IQR]: non-progressors, 104 mL/min/1.73 m^2 [103–105 mL/min/1.73 m^2]; progressors, 81 mL/min/1.73 m^2 [71–89 mL/min/1.73 m^2]), increase in urinary BCAA concentration seems to be associated with diabetes mellitus-ESRD progression regardless of DKD stage.

Myo-inositol is a dominant meso-compound of inositol, is defined as a sugar alcohol, and was previously designated as vitamin B8. In the form of inositol derivatives, inositol composes the eukaryotic cell membrane and works as a secondary messenger including the insulin signaling cascade that regulates insulin resistance [32–34]. The regulation of myo-inositol is deeply related to the kidney, as 80% of myo-inositol is synthesized in the kidney, and the kidney is the sole organ for myo-inositol catabolism by myo-inositol oxygenase, which is a renal tubular-specific enzyme [34,35]. In our results, urinary myo-inositol showed significant increments as DKD stage progressed and was associated with increased risk of ESRD progression even after baseline adjustment. Moreover, our previous study demonstrated that urinary myo-inositol increased in streptozotocin-induced diabetic mice compared with controls and decreased after losartan treatment [21]. Other studies reported intracellular depletion of myo-inositol in diabetic patients and excessive urinary myo-inositol excretion in human and animal studies [36,37]. Given the importance of the kidney in the regulation of myo-inositol and the increased concentrations of urinary myo-inositol observed across studies, myo-inositol can be positively considered as a prognostic biomarker for DKD.

As a secondary messenger in insulin, myo-inositol is thought to have insulin-sensitizing effects and myo-inositol supplementation is a putative treatment for polycystic ovary syndrome [38] and gestational diabetes mellitus [39]. Recently, a study of streptozotocin-induced diabetic mice reported that myo-inositol supplementation ameliorated albuminuria and enhanced renal function by up-regulation of mitophagy proteins and positive modulation of mitochondrial biogenesis [40]. Of course, more cellular, animal, and clinical studies are needed, but treatment options beyond the predictive utility of myo-inositol in DKD patients are possible.

In addition to myo-inositol, other urine metabolites changed according to DKD staged and had ESRD prediction power. Among them, urinary choline concentration increased with DKD stage and showed high ESRD progress predictive power (AUC, 0.770; 95% confidence interval [CI] = 0.71–0.83 [Supplementary Table 4, available online]; 12-month prediction: AUC, 0.834; 95% CI = 0.77–0.89 [Supplementary Table 5, available online]). However, there was no statistical significance when patient's baseline characteristics were adjusted for multivariate Cox analysis (model 3: HR, 1.21, 95% CI = 0.98–1.50, p = 0.07) (Table 3), and there was no additional benefit regarding serum creatinine and UPCR when evaluated with NRI and IDI (NRI: 1.2%, p = 0.82; IDI: 24.6%, p = 0.23).

Although our results are informative, this study has some limitations. First, even though potential precedent mechanisms are discussed above, more detailed mechanical and experimental studies are needed. Second, since we obtained only one test per patient, we did not consider differences between individuals over time. Third, because UACR data were missing for many patients, urinary metabolites could not be compared with UACR but were compared with UPCR. Moreover, as the majority of the participants were advanced DKD patients, it may be difficult to apply our findings to early DKD patients. Fourth, although human urinary metabolomics results can be affected by medication usage, we did not consider mediation usage in the present study due to the difficulty of concurrently adjusting for multidrug usage. Further studies are necessary to analyze urinary metabolites of DKD while adjusting for medications such as metformin, sodium-glucose cotransporter 2 inhibitors, and glucagon-like peptide-1 receptor agonists.

In conclusion, urinary myo-inositol concentrations increased as DKD stage progressed and showed additive effects in predicting ESRD progression when considered alongside the conventional renal dysfunction markers, eGFR and UPCR.

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Conflicts of interest

All authors have no conflicts of interest to declare.

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Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors' contributions

Conceptualization: GSH, JPL
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