Title:

Identification of novel urinary biomarkers for predicting the renal prognosis in patients with type 2 diabetes by glycan profiling in a multicenter prospective cohort study: U-CARE Study 1

Running head:

Novel glycan biomarkers of DKD

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ABSTRACT

OBJECTIVE Because quantifying glycans with complex structures is technically challenging, little is known about the association of glycosylation profiles with the renal prognosis in diabetic kidney disease (DKD).

RESEARCH DESIGN AND METHODS In 675 patients with type 2 diabetes, we assessed the baseline urinary glycan signals binding to 45 lectins with different specificities. The endpoint was a decrease of estimated glomerular filtration rate (eGFR) by ≥ 30% from baseline or dialysis for end-stage renal disease.

RESULTS During a median follow-up period of 4.0 years, 63 patients reached the endpoint. Cox proportional hazards analysis revealed that urinary levels of glycans binding to 6 lectins were significantly associated with the outcome after adjustment for known indicators of DKD, although these urinary glycans except that for DBA were highly correlated with baseline albuminuria and eGFR. Hazard ratios for these lectins were (+1 SD for the glycan index): SNA (recognizing glycan: Siaα2-6Gal/GalNAc): 1.42 (95%CI: 1.14-1.76), RCA120 (Galβ4GlcNAc): 1.28 (1.01-1.64), DBA (GalNAcα3GalNAc): 0.80 (0.64-0.997), ABA (Galβ3GalNAc): 1.29 (1.02-1.64), Jacalin (Galβ3GalNAc): 1.30 (1.02-1.67), and ACA (Galβ3GalNAc): 1.32 (1.04-1.67). Adding these glycans indexes to a model containing known indicators of progression improved prediction of the outcome (net reclassification improvement increased by 0.51 (0.22-0.80), relative integrated discrimination improvement increased by 0.18 (0.01-0.35), and the Akaike information criterion decreased from 296 to 287).

CONCLUSIONS The urinary glycan profile identified in this study may be useful for predicting the renal prognosis in patients with type 2 diabetes. Further investigation of glycosylation changes and urinary glycan excretion in DKD is needed.

INTRODUCTION

It has been demonstrated that several biomarkers can predict the renal prognosis in patients with diabetes. Blood levels of tumor necrosis factor receptor (TNFR) 1 and 2 are well known prognostic indicators for diabetic kidney disease (DKD) (1,2). Similarly, markers of tubulointerstitial injury, inflammation, and filtration have been reported to predict the progression of DKD (3-6). These biomarkers partly allow us to predict the renal prognosis at an early stage of DKD independently of established clinical factors, such as the estimated glomerular filtration rate (eGFR) and albuminuria. However, despite early detection of high-risk patients and recent new protective treatment for DKD (7-9), it remains difficult to manage patients with DKD and rapid deterioration of renal function. Thus, there is a need to discover new biomarkers in order to identify the pathogenesis of DKD and emerging therapeutic targets.

Recently, the role of glycans and their enzymatic modification ("glycosylation") have attracted much attention in relation to research on cancer and metabolic diseases, including diabetes and DKD. Ohtsubo *et al*. reported that human pancreatic β-cell-specific GnT-4a glycosyltransferase, which generates the core β1-4 GlcNAc linkage in *N*-glycans, had a protective effect against diabetes by increasing glucose transporter 2 (*GLUT2*) expression and maintaining insulin secretion (10). Differences of glycosylation, including *O*-GlcNAcylation, were reported to contribute to the progression of DKD in rats (11,12). There have been few reports about differences of glycosylation in DKD patients because of technical obstacles to glycan analysis due to the complicated structures of these molecules and the time-consuming processes required for mass spectrometry (MS). However, the evanescent-field fluorescence-assisted lectin microarray that we reported previously enables

high-throughput quantification of glycan binding to 45 specific lectins (13,14). In the preliminary analysis of our previous study (14), urinary glycosylation pattern considerably varied, while the changes in serum glycosylation was barely detectable among the patients with different kidney diseases including DKD associated with almost equivalent proteinuria and eGFR. Moreover, a recent study of the serum *N*-glycan profile revealed that differences of *N*-glycosylation were associated with diabetic complications and glycemic control in patients with type 1 and 2 diabetes (15,16), but the association between the urinary glycosylation profile and the renal prognosis has not been investigated in patients with diabetes to the best of our knowledge.

Accordingly, we investigated the relationship between urinary excretion of *O*- and *N*-glycans binding to 45 lectins and the renal prognosis in patients with type 2 diabetes. In addition, we assessed the incremental predictive value of adding promising glycans to a model that contained established clinical variables, including albuminuria and eGFR.

RESEARCH DESIGN AND METHODS

Study Design

This prospective cohort study was initiated in 2012. Among 688 patients with type 2 diabetes admitted to 8 hospitals in Japan from June 2012 to March 2013, 675 patients were eligible for enrollment in this study. Exclusion criteria were a diagnosis of slowly progressive type 1 diabetes during follow-up or a baseline eGFR < 15 mL/min/1.73m^2 (Supplementary Fig. 1). The diagnosis of diabetes was based on Japanese Diabetes Society criteria (17). In addition, 134 volunteers who underwent medical checkups at Okayama Health Foundation in March 2016 and were confirmed to have neither diabetes nor CKD (17,18) were enrolled as controls to compare differences of glycosylation. The protocol of this study was approved by the ethics committee of Okayama University Hospital in June 2012 (identification number: H24-003). This study was registered with the University Hospital Medical Information Network (UMIN) in June 2012 (identification number: UMIN000011525). Written informed consent was obtained from all patients with diabetes, whereas comprehensive anonymous consent was obtained from the controls.

Laboratory parameters and definitions

Urine samples collected in the early morning and stored at baseline (patients: 2012-2013, controls: 2016) were used to measure urinary glycans and urinary albumin levels in 2015-2016. All specimens were aliquoted, stored at -80^{o}C until measurement, and thawed for the first time to perform this study. Average storage period until measurement was 2.1 ± 0.1 years for patients, and 0.9 ± 0.0 years for controls, respectively. As shown in Supplementary Table 1, we demonstrated that the influence of the difference in frozen storage duration on measurement of glycosylation is negligible.

Urinary glycans were measured by the evanescent-field fluorescence-assisted lectin microarray, which is a new method of glycan profiling (13,19-21). In brief, we measured urinary levels of Cy3-labeled glycoproteins that bound to 45 lectins with different specificities, and urinary glycan intensity can be measured in 300 samples in three working days (Supplementary Fig. 2). Cy3 binds to primary amine in principle. Urinary albumin was also labeled by Cy3, although it lacks glycan modification, resulting in minimal background reactivity. Urinary creatinine was also labeled by Cy3, causing background reactivity like albumin. In preliminary experiments, both urinary albumin and creatinine were significantly associated with the intensity of background reactivity [*r* between log(urinary albumin concentration) and log(background intensity [BG-I]): 0.697, and *r* between log(urinary creatinine concentration [UCr]) and log(BG-I): 0.166, Supplementary Fig. 3]. We also observed that the intensity of glycan reactivity (glycan intensity) in 24-hour urine and spot urine samples was more closely correlated with the net glycan intensity [Net-I; raw glycan intensity (Raw-I) – BG-I] than with the Net-I/UCr ratio or the Raw-I/UCr ratio [spot urine/24-hour urine Net-I ratio: 1.20 ± 0.74 (mean ± SD), Net-I/UCr ratio: 1.77 ± 2.69, and Raw-I/UCr ratio: 1.68 ± 2.00] (Supplementary Fig. 4). Furthermore, comparison of glycan intensity between original urine samples and 10-fold diluted urine samples showed that all of the Raw-I ratios [ratio: (original urine intensity)/(10-fold diluted urine intensity)] and most of the Net-I ratios were less than 10, suggesting that the urinary glycan index did not increase linearly, but quadratically (Supplementary Table 2). Based on these results, we performed all analyses by using the glycan index appropriately transformed from the Net-I according to its distribution.

GFR was estimated by using the Japanese coefficient–modified Chronic Kidney

Disease Epidemiology Collaboration equation (22). The baseline urinary albumin/creatinine ratio (UACR; mg/gCr) was measured in a spot urine specimen, and normoalbuminuria, microalbuminuria, and macroalbuminuria were defined as UACR <30 mg/gCr, UACR ≥30 and < 300 mg/gCr, and UACR ≥300 mg/gCr, respectively (15). HbA1c data are presented as National Glycohemoglobin Standardization Program values according to the recommendations of the Japanese Diabetes Society and the International Federation of Clinical Chemistry (23). Body mass index (BMI) was calculated as weight divided by the square of height (kg/m^2). Mean arterial pressure (MAP) was calculated as 2/3 of diastolic pressure + 1/3 of systolic pressure (mmHg). Hypertension was defined as a baseline blood pressure ≥ 140/90 mmHg or use of antihypertensive drugs. The average annual values of clinical parameters including systolic blood pressure (SBP), diastolic blood pressure (DBP), MAP, and HbA1c plus use of an angiotensin-converting enzyme inhibitor (ACE-I) or angiotensin II type I receptor blocker (ARB) during follow-up were compared between patients with and without outcome in all participants and in those stratified according to baseline eGFR categories. The grade of diabetic retinopathy was determined by an ophthalmologist at baseline (24). In this study, cardiovascular disease (CVD), stroke, and peripheral arterial disease (PAD) were defined as events requiring admission for treatment, cerebral bleeding or infarction requiring admission for treatment, and PAD requiring admission for intervention or surgery, respectively. Cardiovascular events were defined as any CVD, stroke, or PAD event.

Study Endpoint

The primary endpoint of the study was defined as a decrease of eGFR by at least 30% from baseline or commencement of dialysis for end-stage renal disease (ESRD). None

of the patients received kidney transplantation during follow-up.

Statistical Analysis

Data were summarized as percentages or as the mean ± standard deviation [SD], as appropriate. All skewed variables were subjected to logarithmic transformation to improve normality before analysis. Correlations among glycan indexes were evaluated by Pearson's correlation analysis. Univariate and multivariate linear regression analyses were employed to explore the association of urinary glycan index with baseline HbA1c and age. In both regression models, dependent variable was glycan index. In each multivariate model, β-coefficient for HbA1c was adjusted for baseline age, gender, and duration of diabetes, while β-coefficient for age was adjusted for gender and duration of diabetes. The cumulative incidence rate of the primary outcome was estimated by drawing Kaplan-Meier curves for urinary glycan quartiles in all patients, and incidence rates were compared with the log-rank test. In addition, we compared Kaplan-Meier curves between groups of patients with normoalbuminuria or microalbuminuria stratified according to urinary glycan quartiles (Q1-3 vs. Q4 or Q1 vs. Q2-4), and also compared groups of patients with macroalbuminuria and higher or lower glycan indexes than the median value. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for the death-censored endpoint. In the multivariate model, HRs were adjusted for age, gender, MAP, HbA1c, eGFR, and log-transformed UACR at baseline. These covariates were selected as potential confounders on the basis of biological plausibility and metabolic memory (25,26). We also examined another multivariate model without baseline HbA1c to evaluate the impact of baseline HbA1c levels on the outcome. Improvement in discriminating the risk of the study outcome at the median follow-up time (4.0 years) was assessed by

analysis of category-free net reclassification improvement (NRI) and absolute/relative integrated discrimination improvement (IDI), as reported elsewhere (27-29). The median follow-up time was selected as the cut-off point for analysis because it was previously employed in similar biomarker studies (3,30). We also used the Akaike information criterion (AIC) to compare the fit of various models. Moreover, we compared Harrell's concordance index (c-index) between multivariate Cox proportional hazards models with or without glycan biomarkers. The 95% CIs for category-free NRI and IDI and the differences of the c-index were computed from 5000 bootstrap samples to adjust for optimism bias. Two-tailed P values < 0.05 were considered to indicate statistical significance. Analyses and creation of graphs were performed with Stata SE software (version 14.0, StataCorp LP) and Origin (version 2017, OriginLab).

RESULTS

Follow-up period and incidence of the outcome

The median follow-up period was 4.0 years (interquartile range [IQR]: 3.9-4.0 years). During follow-up, the primary endpoint occurred in 63 patients (9%) and 12 patients (2%) died from causes other than ESRD after refusing dialysis or transplantation.

Patient Characteristics

The baseline characteristics of the subjects are shown in Table 1. Their age was 63 ± 11 years (mean ±SD), 61% of the patients were men, and the median known duration of diabetes was 11.0 years (IQR: 6.2-17.7). The baseline SBP, DBP, and MAP was 131.0 ± 17.1 mmHg, 74.8 ± 10.9 mmHg, and 93.5 ± 11.7 mmHg, respectively. One third of the patients had diabetic retinopathy (any type) and their mean baseline HbA1c was 7.1 ± 1.1 % (54.3 ± 12.0 mmol/mol). In addition, the mean baseline eGFR was 71.4 ± 17.1 ml/min/1.73m^2, median UACR was 17.3 mg/gCr (IQR: 7.8-71.1), and 594 patients had normoalbuminuria (64%) or microalbuminuria (24%). With respect to blood pressure and glycemic control during follow-up, average SBP, DBP, MAP, and HbA1c were not significantly different between patients with and without outcome, as well as use of ACE-I or ARB during follow-up were not significantly different between the patients with and without outcome in groups stratified according to baseline eGFR categories (Supplementary Table 3).

Associations of urinary glycan index with HbA1c and age

Univariate and multivariate regression analysis in all patients and patients with baseline eGFR ≥60 ml/min/1.73m^2 revealed that lower HbA1c was significantly associated with glycan complex structures that have higher GlcNAc (recognized by PHA(L), PHA(E),

DSA, LEL, STL, and WGA), higher Siaα3Galβ4GlcNAc (recognized by MAL_I, and ACG), higher GalNAcβ4GlcNAc (recognized by WFA), and higher GalNAcα3GalNAc (recognized by DBA), while higher HbA1c was significantly associated with higher Manα3Man (recognized by GNA). Most of these significant associations were attenuated in the patients with baseline eGFR <60 ml/min/1.73m^2 (Supplementary Table 4). Similar regression analysis for age showed that older age was associated with higher Fucα2GalβGlcNAc (recognized by UEA_I), higher (Galβ4GlcNAc)n (polylactosamine, recognized by LEL), higher (GlcNAcβ4MurNAc)n (peptidoglycan backbone, recognized by STL), and higher Siaα3Galβ3(Siaα6)GalNAc (recognized by MAH) (Supplementary Table 5).

Relation between the renal outcome and glycan binding to the lectin panel

Unadjusted and adjusted HRs for glycan binding to the panel of 45 lectins with different specificities and the reported structure of the glycan binding to each lectin are shown in Figure 1. A number of urinary glycans were significantly correlated with the renal outcome in the univariate Cox regression model, while the urinary glycans binding to 6 specific lectins [*Sambucus nigra* (SNA), *Ricinus communis* (RCA120), *Dolichos biflorus* (DBA), *Agaricus bisporus* (ABA), *Artocarpus integrifolia* (Jacalin), and *Amaranthus caudatus* (ACA)] were significantly correlated with the renal outcome in both the univariate and multivariate models. In the multivariate model adjusted for known indicators of DN progression, including baseline eGFR and UACR, the HR for positive glycan binding to SNA (+1 SD for the glycan index) was 1.42 [95% CI: 1.14-1.76], while the HR for glycan binding to RCA120 was 1.28 [1.01-1.64], DBA 0.80 [0.64-0.997], ABA 1.29 [1.02-1.64], Jacalin 1.30 [1.02-1.67], and ACA 1.32 [1.04-1.67] (Figure 1A). These associations remained largely unchanged when average

MAP and/or HbA1c during follow-up period were incorporated into the multivariate model and when baseline HbA1c was eliminated from the multivariate model (Supplementary Table 6 and 7). As shown in Figure 1B, the glycans Siaα2-6Gal/GalNAc, Galβ4GlcNAc, and GalNAcα3GalNAc were reported to bind with SNA, RCA120, and DBA, respectively, whereas Galβ3GalNAc was reported to bind with ABA, Jacalin, and ACA.

Glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA in the control group and the patients with diabetes stratified according to the CKD heat map

We stratified the patients with diabetes into 4 CKD heat map groups and 11 categories using the baseline UACR and eGFR (31). Comparisons of glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA among the control group and patients with diabetes stratified according to the CKD heat map groups and 11 categories are shown in Supplementary Fig. 5A and B. Overall, the glycan index values were higher in the severe categories of CKD heat map, albuminuria, and eGFR, except for the glycan index of DBA. Interestingly, the glycan index of SNA was significantly higher in the green heat map group than in the control group, even though both groups were defined by normoalbuminuria and eGFR >60 mL/min/1.73m^2. Correlations among positive binding to ABA, Jacalin, and ACA were extremely strong (r=0.952 between ABA and Jacalin, r =0.931 between ABA and ACA, and r =0.942 between ACA and Jacalin), as was the correlation between SNA and RCA120 (r=0.921) (Supplementary Table 8).

Cumulative incidence rate of the primary outcome in urinary glycan quartiles

Kaplan-Meier curves stratified according to quartiles for baseline urinary glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA are shown in Figure 2. The cumulative incidence rate of the renal outcome was significantly higher in the highest

quartile for urinary glycan binding to SNA, RCA120, ABA, Jacalin, or ACA than in the other quartiles, whereas it was significantly higher in the lowest quartile for glycan binding to DBA than in the other quartiles (Figure 2A). Similar results for glycans binding to SNA, ABA, Jacalin, and ACA were obtained in patients with normoalbuminuria/microalbuminuria (Figure 2B). Among patients with macroalbuminuria, the cumulative incidence rate was significantly higher in those with higher glycan index values than in those with lower glycan index values, except in the case of the glycan index for DBA (Figure 2C).

Incremental predictive power of urinary glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA

The category-free NRI, absolute/relative IDI, and AIC for predicting the primary outcome at the median follow-up time (4 years) obtained by adding the glycan indexes, as well as the difference of Harrell's c-index between Cox regression models with or without the urinary glycan indexes, are summarized in Table 2. Adding any single glycan index to the multivariate model did not improve prediction, whereas adding all 6 glycan indexes significantly improved risk classification, integrated discrimination, and AIC (category-free NRI: 0.51 [95% CI: 0.22-0.80], relative IDI: 0.18 [0.01-0.35], and AIC decreased from 296 to 287). Similarly, when 4 glycan indexes (based on the reported glycan specificities) were added, risk classification, integrated discrimination, and AIC were also significantly improved (Table 2). However, Harrell's c-index did not increase significantly when these glycan indexes were added to the multivariate Cox regression model (Table 2).

CONCLUSIONS

Glycans are involved in various biological processes, including development, immunity, infection, hormone actions, cell adhesion, and oncogenesis (19,32,33). One of the most prominent features of glycans is “structural heterogeneity”, and this distinguishes them from other major biopolymers such as nucleic acids and proteins. Such heterogeneity is largely attributable to the glycan fabrication process and depends on structural modification by glycosyltransferases, which is known as “glycosylation” (19).

Podocalyxin is the molecule in the kidneys for which the functional effects of changes in glycosylation have been most thoroughly investigated (34). It is a glycoprotein expressed by podocytes that has an essential role in the maintenance of podocyte slit pore integrity and effective glomerular filtration (35). Podocalyxin is decorated by *O*-glycans, including an abundance of sialic acid, and removal of sialic acid leads to effacement of the podocyte foot processes and proteinuria in mice secondary to loss of the negative charge on the glomerular basement membrane (GBM) (36). Similarly, doxycycline-induced global deficiency of glycosyltransferase core 1 synthase and glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase 1 (C1galt1) leads to marked reduction of SNA-binding Siaα2-6Gal/GalNAc on podocalyxin in mice, followed by albuminuria, rapid progression of glomerulosclerosis, effacement of podocyte foot processes, and thickening of the GBM (37). These results might suggest that expression of mature *O*-glycans modified by a key glycosyltransferase in the kidneys, especially on podocytes, is crucial to maintenance of the normal renal architecture. If abnormalities of renal *O*-glycosylation occur, parts of mature *O*-glycans could undergo reduction and be excreted in the urine. While C1galt1 is not involved in modification of Siaα2-6Gal/GalNAc (Supplementary Fig. 6A), deletion of C1galt1

induced a decrease of Siaα2-6Gal/GalNAc and an increase of Tn-antigen in mouse podocalyxin (37), suggesting that impairment of one *O*-glycosyltransferase may also affect other *O*-glycosylation pathways and lead to creation of abnormal *O*-glycans. These suggestions might be supported by our results as follows.

In the present study, urinary levels of glycans binding to SNA, ABA, Jacalin, and ACA were significantly higher in the severe CKD heat map group, and the cumulative incidence rate of the renal outcome was significantly higher in the highest glycan quartiles than in the other glycan quartiles, not only when all patients were investigated but also when only patients with normoalbuminuria/microalbuminuria were assessed (Supplementary Fig. 5, and Figure 2A and B). Importantly, urinary excretion of Siaα2-6Gal/GalNAc binding to SNA was significantly higher in non-CKD patients with diabetes than in non-CKD control subjects without diabetes. This suggests that a change in the glycosylation of Siaα2-6Gal/GalNAc could occur in the early stage of DKD before the onset of albuminuria or detectable deterioration of renal function. Based on these results and the above-mentioned hypothesis that changes of glycosylation may lead to renal structural damage, it might be reasonable that the indexes for these glycans were significantly associated with the risk of the renal outcome independently of baseline albuminuria and eGFR (Figure 1).

Galβ4GlcNAc binds to RCA120 and is involved in *N*- and *O*-glycosylation. During *O*-glycosylation, Galβ4GlcNAc has a role in extension of cores 2 and 4, which are finally modified by sialic acid (32) (Supplementary Fig. 6A). Therefore, if there is impairment of the enzyme adding sialic acid to cores 2 and 4, urinary excretion of Galβ4GlcNAc could increase by the same mechanism as that which increases urinary levels of Siaα2-6Gal/GalNAc and Galβ3GalNAc. In this study, the glycan index for

RCA120 was strongly correlated with that for SNA (r=0.921, Supplementary Table 8), which might suggest that the common underlying mechanism is impairment of sialylation. In addition, Ravida *et al*. demonstrated that glycans binding to RCA120 gradually showed a significant decrease in the renal cortex of diabetic rats with progression of DKD, while these glycans increased in non-diabetic rats (12). Therefore, increased urinary levels of Galβ4GlcNAc might reflect abnormal *O*-glycosylation in kidney tissues.

With regard to GalNAcα3GalNAc, we could not reasonably explain why urinary excretion of this glycan binding to DBA was negatively correlated with the renal outcome. DBA is well-known for recognizing for the epitope of glycans on the surface of type A red blood cells (33). It was also reported to bind to glomerular lesions in human DKD and to the distal tubules in human kidney tissue, but further investigations have not been performed (38,39). DBA recognizes core 5 (GalNAcα3GalNAc), which is a rare component of *O*-glycans, and the relevant glycosyltransferase has not yet been reported (32). Therefore, further investigation of the role of this glycan in the kidneys and DKD is needed.

Taken together, it seems that changes of glycosylation in DKD are strongly associated with the renal prognosis, since abnormal glycosylation might be involved in the progression of DKD. Supplementary Fig. 6 shows a scheme of our hypothesis regarding the mechanism by which abnormalities of glycosylation may be associated with progression of DKD. We also speculate that urinary glycosylation difference well-reflects the local glycosylation changes in kidney tissues rather than alterations of systemic glycosylation based on the discrepancies of glycan profiles between urine and serum/plasma samples (15,16,40). Serum protein *N*-glycan profiling showed that lower

levels of fucosylated biantennary glycans and higher levels of complexes that have more GlcNAc and heavily galactosylated and sialylated glycans were associated with higher HbA1c levels in patients with diabetes (15,16), whereas those associations were not observed in our urinary glycan profiling. Similarly, plasma IgG *N*-glycan profiling revealed that higher levels of bisecting GlcNAc and lower levels of sialylation were correlated with older age, especially age > 60 years (40), while these results were not shown in our study with urine samples. In the preliminary data of our previous study (14), we found the totally different glycosylation patterns between serum and urine samples, as well as different urinary glycosylation among patients of similar degree of proteinuria and eGFR with biopsy-proven kidney diseases including DKD, which might support our speculation. These hypotheses are based on *in vivo* studies and clinical studies of human DKD, so further investigation of glycosylation changes in human kidney tissue is required.

Potential limitations of this study were that the current lectin microarray system does not allow complete determination of glycan structures as MS can, and unknown preferred glycan structure to each lectin might have biased our results. However, this technique is useful for differentiating urinary glycan profiling in individuals, and it enabled us to measure wide range of urinary glycan intensity in high-throughput without liberation process, although conventional methods including MS required both prior liberation and subsequent labeling (19). Another limitation was that this study included patients with various stages of CKD and renal biopsy was not performed in all participants. Therefore, we cannot exclude the possibility that the decline of eGFR in some patients resulted from kidney diseases other than DKD. However, in previous well-known cohort studies of type 2 diabetes, DKD was not always confirmed by renal

biopsy (1,3). Moreover, in the patients with diabetes, renal biopsy is indicated when they demonstrate hematuria, granular casts, sudden onset of nephrotic syndrome, and rapidly progressive glomerulonephritis, whereas such cases were not observed during follow-up in the current study. It is hoped that an ongoing research biopsy study of DKD at our institution might solve this issue in the future.

In conclusion, we demonstrated that urinary excretion of glycans binding to several lectins, including SNA (recognizing Siaα2-6Gal/GalNAc), RCA120 (Galβ4GlcNAc), DBA (GalNAcα3GalNAc), ABA, Jacalin, and ACA (Galβ3GalNAc), were significantly associated with the renal outcome in patients with type 2 diabetes. Adding the combined glycan index to a model with standard risk factors significantly improved prediction of the renal outcome, suggesting that these urinary glycans may be novel predictors of the renal prognosis in patients with type 2 diabetes. In addition, our findings could provide new insights into changes of glycosylation related to DKD. The mechanisms underlying differences of urinary glycan excretion and changes of glycosylation in DKD should be investigated further.

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The contributions of the authors are detailed as follows:

Koki Mise contributed to designing the research, to analysis and interpretation of data, to measuring urinary glycan levels, to collecting and summarizing clinical data, and writing the manuscript.

Mariko Imamura contributed to collecting, summarizing, and assessing clinical data.

Satoshi Yamaguchi contributed to measuring urinary glycan levels, and to collecting and summarizing clinical data.

Sanae Teshigawara contributed to managing patients and assessing data.

Atsuhito Tone contributed to managing patients and assessing data.

Haruhito Uchida contributed to managing patients and assessing data.

Jun Eguchi contributed to managing patients and assessing data.

Atsuko Nakatsuka contributed to managing patients and assessing data.

Daisuke Ogawa contributed to managing patients and assessing data.

Michihiro Yoshida contributed to interpretation of data, and to performing statistical analyses.

Masao Yamada contributed to measuring urinary glycan levels, to interpretation of data, especially urinary glycan data, and to writing the manuscript.

Kenichi Shikata contributed to managing patients and assessing data.

Jun Wada was responsible for the study design, supervised data collection and data analysis, and contributed to drafting and editing of the manuscript.

Prof. Jun Wada is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

M. Yamada was a former emplyee of GP BioSciences Co., Ltd., and is currently an employee of GlycoTechnica Co., Ltd. There are no other relevant declarations relating to employment, consultancy, patents, products in development or marketed products. This does not alter the authors' adherence to all *Diabetes Care* policies on sharing data and materials.

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Table 1. Baseline clinical parameters

Clinical parameters		All patients (n=675)
Male (%)		61
Age (years)		63 ± 11
BMI (kg/m^2)		25.6 ± 4.6
Duration of DM (years)*		11.0 (6.2-17.7)
SBP (mmHg)		131.0 ± 17.1
DBP (mmHg)		74.8 ± 10.9
MAP (mmHg)		93.5 ± 11.7
Hypertension (%)†		70
Retinopathy (NDR/SDR/prePDR/PDR, %)		67/17/6/10
sCr (mg/dl)		0.85 ± 0.35
eGFR (ml/min/1.73m^2)		71.4 ± 17.1
CKD GFR Categories (G1/G2/G3a/G3b/G4, %)		10/70/12/6/3
UACR (mg/gCr)*		17.3 (7.8-71.1)
Normo/Micro/Macro (%)		64/24/12
HbA1c	(%)	7.1 ± 1.1
	(mmol/mol)	54.3 ± 12.0
Triglyceride (mg/dl)*		116 (81-162)
Total cholesterol (mg/dl)		180.5 ± 32.1
LDL cholesterol (mg/dl)		100.0 ± 25.4
Uric acid (mg/dl)		5.3 ± 1.4
Any type of antihypertensive agents (%)		62
ACE-I or ARB (%)		53
Calcium channel blocker (%)		38
Number of antihypertensive agents*		1 (0-2)
Treatment for diabetes (Diet only/OHA/Insulin, %)		4/64/32
Drug treatment for hyperglycemia (SU/GLIN/BG/αGI/TZD/DPP4-I/GLP1, %)		32/10/35/28/15/49/7
Drug treatment for dyslipidemia (%)		65
Drug treatment for hyperuricemia (%)		10
Prior CVD/Stroke/PAD		17/10/2
Prior cardiovascular event (%)		28

Abbreviations; BMI, body mass index; Duration of DM, estimated duration of diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Retinopathy, diabetic retinopathy; NDR/SDR/prePDR/PDR, non-diabetic retinopathy, simple diabetic retinopathy, pre proliferative diabetic retinopathy, and proliferative diabetic retinopathy, respectively; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; CKD GFR Categories G1 ≥90 ml/min/1.73m^2, G2 60-90 ml/min/1.73m^2, G3a 45-59 ml/min/1.73m^2, G3b 30-44 ml/min/1.73m^2, G4 15-29 ml/min/1.73m^2; UACR, urinary albumin creatinine ratio; Normo/Micro/Macro, normoalbuminuria, microalbuminuria, and macroalbuminuria, respectively; LDL cholesterol, low-density lipoprotein cholesterol; ACE-I or ARB, treatment with an angiotensin-converting enzyme inhibitor or angiotensin II type I receptor blocker, respectively; Diet only, diet regimen only; OHA, oral hypoglycemic agent; Insulin therapy, treatment with insulin (including basal-supported oral therapy); SU, sulfonylurea; GLIN, meglitinide anologs; BG, biguanide (Metformin); αGI, alpha-glucosidase inhibitors; TZD, thiazolidinediones; DPP4-I, DPP-4 inhibitors; GLP1, glucagon-like peptide 1 agonists; CVD, cardiovascular disease requiring admission for treatment; Stroke, cerebral bleeding or infarction requiring admission for treatment; PAD, peripheral arterial disease requiring admission for intervention or surgery; Cardiovasular event, any event of CVD, Stroke, and PAD; *Median (interquartile range). †Hypertension was defined as blood pressure ≥ 140/90 mmHg or any antihypertensive drug treatment.

Table 2. Category-free NRI, IDI, and AIC for predicting the 4-year outcome with glycan index data, and difference of Harrell's c-index between Cox regression models with or without glycan index data.

	Category-free NRI (95% CI)	*P*-value	Absolute IDI (95% CI)	*P*-value	Relative IDI (95% CI)	*P*-value	AIC	C-index (95% CI)	Difference of c-index (95%CI)	*P*-value
Only covariates							295.9	0.89 (0.84 - 0.93)		
Glycan to SNA (Siaα2-6Gal/GalNAc)	0.27 (-0.13 - 0.66)	0.184	0.02 (-0.01 - 0.05)	0.184	0.06 (-0.04 - 0.17)	0.232	292.5	0.89 (0.84 - 0.94)	0.00 (-0.01 - 0.01)	0.958
Glycan to RCA120 (Galβ4GlcNAc)	0.02 (-0.25 - 0.29)	0.891	0.00 (-0.01 - 0.02)	0.712	0.01 (-0.03 - 0.05)	0.725	297.6	0.89 (0.84 - 0.93)	-0.00 (-0.01 - 0.00)	0.491
Glycan to DBA (GalNAcα3GalNAc)	0.20 (-0.11 - 0.50)	0.203	0.01 (-0.01 - 0.02)	0.488	0.02 (-0.04 - 0.07)	0.529	295.8	0.89 (0.85 - 0.94)	0.00 (-0.00 - 0.01)	0.402
Glycan to ABA (Galβ3GalNAc)	0.26 (-0.10 - 0.61)	0.156	0.01 (-0.01 - 0.04)	0.309	0.04 (-0.04 - 0.12)	0.336	295.1	0.89 (0.84 - 0.94)	0.00 (-0.01 - 0.01)	0.801
Glycan to Jacalin (Galβ3GalNAc)	0.11 (-0.18 - 0.40)	0.439	0.01 (-0.01 - 0.04)	0.375	0.04 (-0.05 - 0.12)	0.383	295.7	0.89 (0.84 - 0.94)	0.00 (-0.01 - 0.01)	0.834
Glycan to ACA (Galβ3GalNAc)	0.13 (-0.17 - 0.44)	0.388	0.01 (-0.01 - 0.04)	0.286	0.04 (-0.04 - 0.13)	0.311	294.4	0.89 (0.84 - 0.94)	0.00 (-0.01 - 0.01)	0.975
Combination of 4 types of glycan (SNA, RCA120, DBA, and ABA)	0.39 (0.11 - 0.67)	0.006	0.06 (0.01 - 0.10)	0.009	0.17 (0.01 - 0.33)	0.037	284.9	0.90 (0.85 - 0.94)	0.01 (-0.01 - 0.03)	0.351
Combination of 4 types of glycan (SNA, RCA120, DBA, and Jacalin)	0.42 (0.15 - 0.69)	0.002	0.06 (0.01 - 0.10)	0.01	0.17 (0.01 - 0.33)	0.033	284.9	0.90 (0.85 - 0.94)	0.01 (-0.01 - 0.03)	0.441

Combination of 4 types of glycan (SNA, RCA120, DBA, and ACA)	0.46 (0.20 - 0.71)	<0.001	0.06 (0.01 - 0.10)	0.009	0.17 (0.02 - 0.33)	0.027	284.5	0.90 (0.85 - 0.94)	0.01 (-0.01 - 0.03)	0.402
Combination of all glycans	0.51 (0.22 - 0.80)	0.001	0.06 (0.02 - 0.10)	0.008	0.18 (0.01 - 0.35)	0.036	287.0	0.90 (0.85 - 0.94)	0.01 (-0.01 - 0.03)	0.447

Covariates were age, gender, mean arterial pressure, HbA1c, estimated glomerular filtration rate, and log-transformed urinary albumin excretion at baseline. Abbreviations; NRI, net reclassification improvement; IDI, integrated discrimination improvement; AIC, Akaike's information criterion; 95% CI, 95% confidence interval; c-index, concordance index; SNA, *Sambucus nigra*; RCA120, *Ricinus communis*; DBA, *Dolichos biflorus*; ABA, *Agaricus bisporus*; Jacalin, *Artocarpus integrifolia*; ACA, *Amaranthus caudatus*.

Figure Legends

Figure 1. Univariate and multivariate Cox proportional hazard models of the renal outcome and reported glycans binding to 45 lectins. **A**. Cox proportional hazard models. In the multivariate model, HR was adjusted for age, gender, mean arterial pressure, HbA1c, eGFR, and log-transformed urinary albumin excretion at baseline. Renal outcome was defined as 30% eGFR decline or dialysis due to end-stage renal disease. Abbreviations; HR: hazard ratio, 95% CI: 95% confidence interval, eGFR: estimated glomerular filtration rate. **B**. Preferred glycan structures binding to 45 lectins with different specificity.

Figure 2. Cumulative incidence rate of the renal outcome. **A**. Cumulative incidence rate in all patients stratified by urinary glycan quartiles. The estimated 4-year renal failure rate was 25%, 22%, 25%, 23%, and 26% in patients from the highest glycan quartiles for SNA, RCA120, ABA, Jacalin, and ACA, respectively, while it was 15% in patients from the lowest glycan quartile for DBA. The cumulative incidence rate was significantly higher in the highest glycan quartiles for SNA, RCA120, ABA, Jacalin, and ACA than in other quartiles ($P<0.001$), whereas it was significantly higher in the lowest glycan quartile for DBA than in the other quartiles ($P<0.05$). **B**. Cumulative incidence rate in the highest urinary glycan quartiles and other glycan quartiles combined in patients with normoalbuminuria or microalbuminuria. The cumulative incidence rate was significantly higher in the highest glycan quartile (Q4) for SNA, ABA, Jacalin, and ACA than in other quartiles combined (Q1-3 vs. Q4: $P=0.026$ for

SNA, P=0.028 for ABA, P=0.019 for Jacalin, and P=0.002 for ACA). On the other hand, the difference of the cumulative incidence rate between Q4 and Q1-3 was not significant for RCA120 and DBA (Q1-3 vs. Q4: P=0.433 for RCA120, P=0.270 for DBA). **C.** Cumulative incidence rate in patients with macroalbuminuria and higher or lower glycan indexes than the median. The cumulative incidence rate was significantly higher in patients with higher glycan indexes than in those with lower glycan indexes (P<0.001), except the glycan index for DBA (P=0.186). Outcome: ≥30% decline of estimated glomerular filtration rate or dialysis due to end-stage renal disease. The log-rank test was used for failure analysis. Abbreviations: SNA, *Sambucus nigra*; RCA120, *Ricinus communis*; DBA, *Dolichos biflorus*; ABA, *Agaricus bisporus*; Jacalin, *Artocarpus integrifolia*; ACA, *Amaranthus caudatus*.