Urinary A- and C-megalin predict progression of diabetic kidney disease: an exploratory retrospective cohort study

Tomomichi Iida [a,b], Michihiro Hosojima [c,*], Hideyuki Kabasawa [c], Keiko Yamamoto-Kabasawa [d], Sawako Goto [a], Takahiro Tanaka [e], Nobutaka Kitamura [e], Mitsutaka Nakada [f], Shino Itoh [f], Shinya Ogasawara [f], Ryohei Kaseda [b], Yoshiki Suzuki [b], Ichiei Narita [b], Akihiko Saito [a,**]

[a] *Department of Applied Molecular Medicine, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8510, Japan*
[b] *Division of Clinical Nephrology and Rheumatology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8510, Japan*
[c] *Department of Clinical Nutrition Science, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8510, Japan*
[d] *Department of Health Promotion Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8510, Japan*
[e] *Clinical and Translational Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8520, Japan*
[f] *Diagnostics Research Department, Life Innovation Research Institute, DENKA Innovation Center, Denka Co., Ltd., 3-5-1 Asahimachi, Machida, Tokyo 194-8560, Japan*

ABSTRACT

Aims: Megalin, a proximal tubular endocytosis receptor, is excreted in urine in two forms: ectodomain (A-megalin) and full-length (C-megalin). We explored whether urinary megalin levels can be used as independent prognostic biomarkers in the progression of diabetic kidney disease (DKD).
Methods: The associations between baseline urinary A-megalin/creatinine (Cr) and/or C-megalin/Cr levels and the subsequent estimated glomerular filtration rate (eGFR) slope were analyzed using a generalized estimating equation. Patients were categorized into higher or lower groups based on the optimal cutoff values, obtained from a receiver operating characteristic curve, of the two forms of urinary megalin.
Results: We retrospectively analyzed 188 patients with type 2 diabetes. The eGFR slopes of the higher A-megalin/Cr and higher C-megalin/Cr groups were -0.904 and -0.749 ml/min/1.73 m^2/year steeper than those of the lower groups, respectively. Moreover, the eGFR slope was -1.888 ml/min/1.73 m^2/year steeper in the group with both higher A- and higher C-megalin/Cr than in the other group. These results remained significant when adjusted for known urinary biomarkers (albumin, α_1-microglobulin, β_2-microglobulin, and *N*-acetyl-β-D-glucosaminidase).
Conclusions: Urinary A- and C-megalin/Cr levels are likely to be prognostic biomarkers in the progression of DKD independent of other urinary biomarkers.

1. Introduction

Diabetic kidney disease (DKD) is a leading cause of end-stage kidney disease worldwide.[1,2] Although albuminuria is a widely recognized biomarker of the clinical development of DKD,[1,3] it was recently recognized that progressive renal decline can develop in many patients with type 2 diabetes independently of albuminuria.[4,5] The precise mechanism underlying the development and progression of DKD is also largely unknown. However, "proximal tubulopathy" has been recognized as a prime trigger of the disease.[6] Because the proximal tubule is the major site of renal metabolism, metabolic load and resultant derangements in proximal tubular epithelial cells (PTECs) are likely to be

associated with the pathogenesis of DKD.[7–10] Thus, the identification of biomarkers that reflect proximal tubulopathy would help to predict DKD progression.[11]

Megalin, a large (~600 kDa) glycoprotein member of the low-density lipoprotein receptor family,[12] plays a pivotal role in the endocytosis of diverse glomerular-filtered substances into PTECs.[13] In a high-fat-diet–induced, obesity-related murine model of DKD, megalin internalizes pathologic proteins such as free fatty acid-enriched albumin into PTECs, leading to qualitative and quantitative protein metabolic overload in the *endo*-lysosomal system and to increased production of platelet-derived growth factor-B and monocyte chemoattractant protein-1 in cells. Subsequent interstitial fibrosis, peritubular capillary rarefaction, and tubular constriction appear to cause retrograde glomerular damage.[9] Advanced glycation end products are also filtered by glomeruli and reabsorbed *via* megalin by PTECs, causing glycotoxicity in diabetes.[14] Thus, megalin appears to constitute a "gateway" for internalizing lipotoxic and glycotoxic substances into the kidney for the development and progression of DKD. Furthermore, several urinary biomarkers filtered by glomeruli (*e.g.*, α_1-microglobulin [α_1-MG], β_2-microglobulin [β_2-MG], liver-type fatty acid-binding protein, neutrophil gelatinase-associated lipocalin, as well as albumin) are known endocytic ligands of megalin.[15,16]

We previously established a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the ectodomain (A-megalin) and full-length (C-megalin) forms of urinary megalin using monoclonal antibodies against the amino- and carboxyl-terminals of megalin, respectively.[17] Compared with normal controls, urinary C-megalin excretion was found to be elevated even in the normoalbuminuric stage of patients with type 2 diabetes and to increase in conjunction with DKD progression in a cross-sectional analysis.[17] We also found that urinary C-megalin is increased *via* exocytosis in association with megalin-mediated quantitative or qualitative protein metabolic load to the *endo*-lysosomal system of PTECs in residual nephrons in type 2 diabetes.[18] In contrast, urinary A-megalin was found to be increased in normo- and micro-albuminuric patients with type 2 diabetes but not in those with macroalbuminuria,[17] and its urinary excretion appears to be regulated by the intracellular recycling[19] and extracellular cleavage[17] of megalin. Thus, the two forms of urinary megalin excretion may play discriminative roles as biomarkers for DKD. However, it is currently unknown whether urinary megalin can longitudinally and independently predict the prognosis of DKD. Furthermore, our previous method for measuring A-megalin in urine is complicated and time-consuming.[17] Therefore, we developed and validated a novel method for measuring urinary A-megalin.

To evaluate the progression of DKD, clinical studies have been designed using endpoints of end-stage kidney disease, renal death, or treatment with renal replacement therapies.[20,21] However, these endpoints need a very long follow-up period and are costly.[22] For these reasons, recently, the estimated glomerular filtration rate (eGFR) slope has been recognized as a surrogate endpoint of chronic kidney disease (CKD) in clinical trials.[23–25] Several studies reported that the eGFR slope of cohorts could predict the progression of CKD, including DKD.[26–31] However, sodium glucose cotransporter 2 (SGLT2) inhibitors cause an initial drop in eGFR and may affect the annual assessment of the eGFR slope.[32] Hence, in this exploratory study, we examined the potential of urinary A- and C- megalin to act as prognostic biomarkers, independent of other known urinary biomarkers, in the progression of DKD in type 2 diabetic patients not taking SGLT2 inhibitors.

2. Methods

2.1. Participants and study design

This retrospective study involved 191 Japanese adults (aged 20 years or older) with type 2 diabetes who were outpatients at the Division of Clinical Nephrology and Rheumatology, Niigata University Medical and Dental Hospital. Urine samples were collected from 2007 to 2011 with their consent. This protocol was approved by the Niigata University Ethical Committee (approval no. 191 in 2003). Patients with severe active infectious disease, severe trauma, or pregnancy were excluded. Patients in the perioperative period or with a short observation period (<6 months) were also excluded. Standard clinical examinations and biochemical tests were performed regularly in the patients, and urine samples were stocked annually at −80 °C during the follow-up period. The oldest stocked urine sample of all participants collected during this period was defined as the baseline sample. We measured urine A-megalin, C-megalin, α_1-MG, β_2-MG, *N*-acetyl-β-D-glucosaminidase (NAG), albumin, and creatinine (Cr) levels. Serum Cr, blood urea nitrogen, uric acid, and hemoglobin A1c of blood samples collected on the same date were measured as baseline data. The eGFR slope was calculated using eGFR values derived from serum Cr levels, which were recorded about once a year in the electronic medical records and collected retrospectively. The permissible ranges for data collection at 1, 2, 3, and 4 years after the baseline day were 6–17 months, 18–29 months, 30–41 months, and 42–53 months, respectively.

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the Niigata University Ethical Committee (approval no. 2591 in 2016). In addition, to validate our novel method for measuring urinary A-megalin, we enrolled 34 patients with type 2 diabetes treated at the Division of Nephrology and Rheumatology in Niigata University Medical and Dental Hospital; this was approved by the Niigata University Ethical Committee (approval no. 2590 in 2016). All participants provided informed consent and their anonymity was preserved. This study was retrospectively registered with the University Hospital Medical Information Network–Clinical Trials Registry (UMIN-CTR) on October 29, 2021 (registration ID, UMIN000045908).

2.2. Measurement of human megalin in urine by sandwich ELISA

Urinary C-megalin was measured as reported previously.[17] A novel sandwich ELISA for measuring urinary A-megalin was developed as follows. The capture monoclonal antibody A12 (7 μg/ml), which was raised against the NH$_2$-terminal ligand-binding domain 1 (LDB1) of human megalin,[17] was immobilized on ELISA plates (F16 Black Maxisorp FluoroNunc Cert; Thermo Fisher Scientific, Waltham, MA) (100 μl/well in 50 mmol/l carbonate buffer, pH 9.5) at 4 °C overnight. The plates were washed with Tris-buffered saline (25 mmol/l Tris-HCl, 137 mmol/l NaCl, 2.68 mmol/l KCl, pH 7.4), blocked with 0.2 % casein and 0.05 % Tween 20 (200 μl/well) in Tris-buffered saline containing 0.1 % NaN$_3$ at 4 °C overnight, and stored at 2 °C–8 °C. The tracer monoclonal antibody A5, which was also raised against the LBD1,[17] was digested by pepsin to prepare secondary fragment antibodies [F(ab')$_2$] that were reduced to F(ab') by 2-mercaptoethylamine. The reduced F(ab') were conjugated to alkaline phosphatase (Roche Diagnostics). Urine samples (50 μl) were mixed with 50 μl of solution B (400 mmol/l Tris-HCl, 40 mmol/l ethylenediaminetetraacetic acid, 2 % Triton X-100, 0.05 % reduced glutathione, pH 8.0) and incubated for 1 min at room temperature. Urine sample mixtures were then reacted with the alkaline phosphatase-labeled tracer monoclonal antibody in the ELISA plates. A chemiluminescent immunoassay detection system with CDP-Star substrate with Emerald-II enhancer (Applied Biosystems, Carlsbad, CA) was used according to the manufacturer's instructions (ELISA-Light System; Thermo Fisher Scientific). A recombinant fusion protein of the LBD1 and cytoplasmic tail of human megalin was used as the calibration standard for measuring both urinary A- and C-megalin, as reported previously.[17] Urinary megalin concentrations were standardized by adjustment to urinary Cr concentrations. The novel method for urinary A-megalin measurement was validated using urine samples of 34 patients with type 2 diabetes by comparison with the previously reported method.[17]

2.3. Measurement of other urinary biomarkers

Serum Cr concentrations were measured by an enzymatic method. The eGFR was calculated with an equation validated for the Japanese population.[33] Urinary concentrations of Cr, albumin, NAG, α_1-MG, and β_2-MG were measured as reported previously.[17] The concentrations of each urinary biomarker were normalized to those of urinary Cr.

2.4. Statistical analysis

We compared our novel method for urinary A-megalin measurement with our previous method using Spearman's correlation analysis. The correlations of baseline urinary biomarkers, including urinary megalin, and clinical parameters were analyzed using Pearson's or Spearman's correlation analysis.

The eGFR slope of each patient was calculated using a generalized estimating equation (GEE).[34] A receiver operating characteristic (ROC) curve was plotted, and the area under the ROC curve was calculated to evaluate the optimal cutoff values of urinary A- or C-megalin/Cr for the median of the eGFR slopes from baseline to visit 4. After patients were categorized into two groups according to the optimal cutoff or median values of the urinary A- or C-megalin/Cr levels ("higher A-megalin/Cr group" *vs.* "lower A-megalin/Cr group" and "higher C-megalin/Cr group" *vs.* "lower C-megalin/Cr group"), their baseline characteristics were compared using Welch's *t*-test, Mann-Whitney's *U* test, or chi-square test. For evaluation of the association between baseline urinary A- or C-megalin/Cr and the eGFR change from baseline, a GEE[34] was used by analyzing time, baseline urinary A- or C-megalin/Cr, and the interaction between time and baseline urinary A- or C-megalin/Cr as independent variables and the eGFR change from baseline to each time as a dependent variable. In this analysis, we determined whether the interaction between the time and baseline urinary A- or C-megalin/Cr is related to the difference in eGFR slopes between higher and lower A- or C-megalin/Cr groups. In addition to the crude model (model 1), models were adjusted with the urinary albumin creatine ratio (ACR) (model 2), urinary α_1-MG/Cr (model 3), urinary β_2-MG/Cr (model 4), and urinary NAG/Cr (model 5). Then, the baseline characteristics of the groups were compared, and changes from baseline were similarly evaluated with a generalized linear model by using a GEE. These models, which were and were not adjusted for other known urinary biomarkers were designed to demonstrate the independence of urinary megalin.

We also categorized the participants into two groups, a "higher megalin/Cr group" and a "lower megalin/Cr group". The participants in the higher megalin/Cr group had both a higher urinary A-megalin/Cr ($\geq$cutoff point or median) and higher urinary C-megalin/Cr ($\geq$cutoff point or median). The lower megalin/Cr group included all participants except those in the higher megalin/Cr group. In addition, we evaluated the difference in the eGFR slope between the higher and lower megalin groups by using a GEE, as described above.

All statistical analyses were performed using SAS Statistics version 9.4 (SAS Institute Inc., Cary, NC), R version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria), or IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY). The level of significance was $P < 0.05$.

3. Results

3.1. Validation of the novel sandwich ELISA for measuring urinary A-megalin

The urine samples of 34 patients with type 2 diabetes were used to validate our novel deoxidization method for measuring urinary A-megalin levels. The profiles of these patients are shown in Appendix A. Each urine sample was measured three times by our previous heating method and our novel deoxidization method. The mean absorbance values and the standard deviation are shown in Appendix B.1 and the concentrations, which were calibrated to the standard curve, are plotted

in Appendix B.2. The two methods were strongly correlated ($r = 0.91$, $P < 0.001$). Therefore, we adopted the novel method for this study.

3.2. Recruitment of patients with type 2 diabetes

In this study, we measured urinary A- and C-megalin using urine samples stocked from 2007 to 2011 from 191 patients with type 2 diabetes. Three patients were excluded according to the exclusion criteria. Therefore, 188 patients were analyzed in this study (Table 1). The distribution of baseline eGFR and albuminuria in the 188 patients is shown in Appendix C. The median observation period was 1456 (interquartile range, 793–1505) days. None of the patients were started on renal replacement therapy during the observation period. The drugs administered to the patients are listed in Appendix D. No patient had been treated with SGLT2 inhibitors.

3.3. Cutoff points of urinary A- and C-megalin excretion levels from ROC analysis

ROC curves were analyzed to predict the eGFR slope (-1.013 ml/min/1.73 m^2/year). With a cutoff value of 222.52 pmol/g Cr, the area under the ROC curve showed that urinary A-megalin/Cr had specificity and sensitivity of 46 % and 68 %, respectively, for an eGFR slope > -1.013 ml/min/1.73 m^2/year. With a cutoff value of 0.62 pmol/g Cr, the area under the ROC curve also showed that urinary C-megalin/Cr had specificity and sensitivity of 48 % and 70 %, respectively, for an eGFR slope > -1.013 ml/min/1.73 m^2/year. Patients with urinary A- or C-megalin/Cr greater than or equal to these cutoff points were respectively classified as the higher A- or higher C-megalin/Cr groups, whereas those with values less than these cutoff points were classified as the lower A- or lower C-megalin/Cr groups.

3.4. Longitudinal analysis of the association of urinary A- or C-megalin excretion with eGFR slopes

The baseline clinical characteristics of the higher and lower A- or C-megalin/Cr groups are shown in Appendix E.1 and .2, respectively. Each eGFR slope analyzed from the generalized linear model is shown in Table 2, Appendix E.3, Table 3, Appendix E.4 and Fig. 1. Tables 2 and 3 show that the interaction between time and baseline urinary A- or C-

Table 1

Baseline parameters and other data of the 188 patients analyzed in this study.

Number (male/female)	104/84
Age (years)	66.3 ± 11.6
BMI	25.36 ± 4.95
SBP (mmHg)	131 ± 14.8
eGFR (ml/min/1.73 m^2)	67.6 ± 21.4
BUN (mg/dl)	17.7 ± 9.1
S-Cr (mg/dl)	0.90 ± 0.48
S-UA (mg/dl)	5.5 ± 1.4
HbA1c (%)	7.3 ± 0.9
U–A-megalin/Cr (pmol/g Cr)	176.25 ± 134.63
U–C-megalin/Cr (pmol/g Cr)	0.81 ± 1.47
U-ACR (mg/g Cr)	252.7 ± 666.6
U–α_1-MG/Cr (mg/g Cr)	13.79 ± 16.12
U–β_2-MG/Cr (μg/g Cr)	1120.84 ± 5489.29
U-NAG/Cr (IU/g Cr)	8.52 ± 6.65
RAS inhibitors	126

Data are number or mean ± standard deviation. BMI, body mass index; SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; S—Cr, serum creatinine; S-UA, serum uric acid; HbA1c, hemoglobin A1c; U–A-megalin, urinary A-megalin; U–C-megalin, urinary C-megalin; U-ACR, urinary albumin creatinine ratio; U–α_1-MG, urinary α_1-microglobulin; U–β_2-MG, urinary β_2-microglobulin; U-NAG, urinary *N*-acetyl-β-D-glucosaminidase; RAS, renin-angiotensin-aldosterone system.
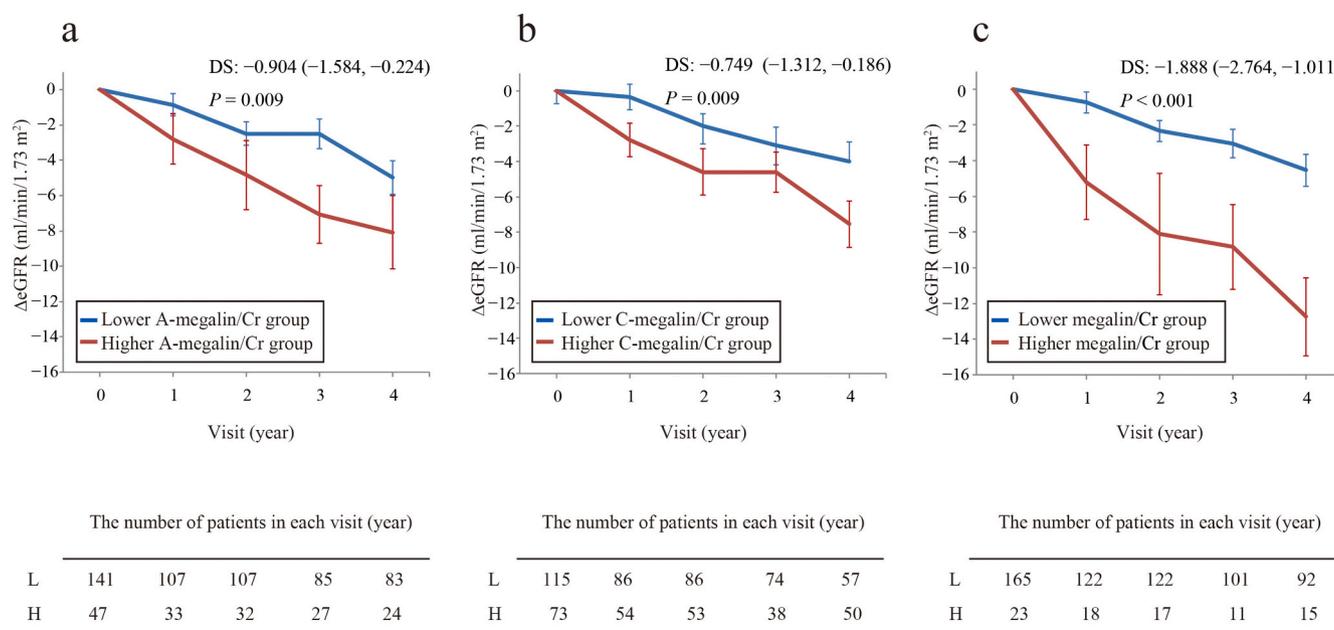
Fig. 1. Estimated glomerular filtration rate (eGFR) decline at each urinary megalin/Cr level.
Each graph shows the results of the crude model or model 1 of urinary A-megalin/Cr, urinary C-megalin/Cr, and urinary megalin/Cr for the differences in eGFR slopes presented in Tables 2, 3 and 4, respectively. Each plot shows the mean eGFR of each visit. The error bars show the standard error. The table below each graph shows the participant numbers for each visit. (a) eGFR transition plotted by urinary A-megalin/Cr level divided by the cutoff point. The red line shows the higher A-megalin/Cr group. The blue line shows the lower A-megalin/Cr group. (b) eGFR transition plotted by urinary C-megalin/Cr level divided by the cutoff point. The red line shows the higher C-megalin/Cr group. The blue line shows the lower C-megalin/Cr group. (c) eGFR transition plotted by urinary megalin/Cr level divided by the cutoff point. The red line shows the higher megalin/Cr group. The participants in the higher megalin/Cr group had both a higher urinary A-megalin/Cr ($\geq$222.52 pmol/g Cr) and higher urinary C-megalin/Cr ($\geq$0.62 pmol/g Cr). The blue line shows the lower megalin/Cr group that included all participants except those in the higher megalin/Cr group. Cr, creatinine; H, higher A-megalin/Cr group or C-megalin/Cr group or megalin group; L, lower A-megalin/Cr group or C-megalin/Cr group or megalin group; DS, difference of slopes.

Table 2
Longitudinal analysis of the association of urinary A-megalin excretion with the eGFR slope.

Model	Difference in eGFR slopes (ml/min/1.73 m^2/year)	P-value
U–A-meg/Cr	−0.904 (−1.584, −0.224)	0.009
U–A-meg/Cr + ACR	−1.005 (−1.698, −0.312)	0.004
U–A-meg/Cr + U–α_1-MG/Cr	−0.990 (−1.678, −0.303)	0.005
U–A-meg/Cr + U–β_2-MG/Cr	−0.922 (−1.603, −0.242)	0.008
U–A-meg/Cr + U-NAG/Cr	−0.989 (−1.682, −0.295)	0.005

For evaluation of the association between baseline urinary A-megalin/Cr and the eGFR change from baseline, a generalized estimating equation (GEE) was used by analyzing time, baseline urinary A-megalin/Cr, and the interaction between time and baseline urinary A-megalin/Cr as independent variables and the eGFR change from baseline to each time as a dependent variable. Here, the table shows whether the interaction between the time and the baseline urinary A-megalin/Cr is related to the difference in eGFR slopes. The difference in slopes represents the difference in eGFR slopes and the 95 % confidence interval between the higher and lower A-megalin/Cr groups divided by the cutoff point. Each model was analyzed either as a crude model or after adjustment for the known urinary biomarkers listed. U–A-meg, urinary A-megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U–α_1-MG, urinary α_1-microglobulin; U–β_2-MG, urinary β_2-microglobulin; U-NAG, urinary N-acetyl-β-D-glucosaminidase.

megalin/Cr are related to the difference in the eGFR slope. We have also presented tables showing the original statistical analyses in Appendix E.3 and .4. The eGFR slope was −0.904 (95 % CI −1.584, −0.224) ml/min/1.73 m^2/year steeper in the higher A-megalin/Cr group than in the lower A-megalin/Cr group in the crude model or model 1 (Table 2, Appendix E.3, and Fig. 1). Moreover, in the models adjusted with other

Table 3
Longitudinal analysis of the association of urinary C-megalin excretion with the eGFR slope.

Model	Difference of eGFR slopes (ml/min/1.73 m^2/year)	P-value
U–C-meg/Cr	−0.749 (−1.312, −0.186)	0.009
U–C-meg/Cr + ACR	−0.827 (−1.403, −0.250)	0.005
U–C-meg/Cr + U–α_1-MG/Cr	−0.826 (−1.401, −0.251)	0.005
U–C-meg/Cr + U–β_2-MG/Cr	−0.768 (−1.332, −0.203)	0.008
U–C-meg/Cr + U-NAG/Cr	−0.989 (−1.444, −0.276)	0.004

The same analysis was conducted for urinary C-megalin/Cr as described in the footnote to Table 2. U–C-meg, urinary C-megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U–α_1-MG, urinary α_1-microglobulin; U–β_2-MG, urinary β_2-microglobulin; U-NAG, urinary N-acetyl-β-D-glucosaminidase.

urinary biomarkers, the slopes showed a similar trend (Table 2 and Appendix E.3). Also, the eGFR slope was −0.749 (95 % CI −1.312, −0.186) ml/min/1.73 m^2/year steeper in the higher C-megalin/Cr group than in the lower C-megalin/Cr group in the crude model or model 1 (Table 3, Appendix E.4, and Fig. 1). Furthermore, in models adjusted with other urinary biomarkers, the slopes showed a similar trend (Table 3 and Appendix E.4).

According to the median of each urinary megalin level at baseline, these 188 patients were also categorized into higher and lower A- or C-megalin/Cr groups. The characteristics of these groups are shown in Appendix F.1 and .2, respectively. Each eGFR slope analyzed from the generalized linear model is shown in Appendix F.3 and .4. The eGFR slope was −0.749 (95 % CI −1.297, −0.202) ml/min/1.73 m^2/year steeper in the higher A-megalin/Cr group than in the lower A-megalin/

Table 4

Longitudinal analysis of the association of urinary A- and C-megalin excretion with the eGFR slope.

Model	Difference of eGFR slopes (ml/min/1.73 m^2/ years)	*P*-value
U-meg/Cr	−1.888 (−2.764, −1.011)	<0.001
U–meg/Cr + ACR	−1.951 (−2.836, −1.065)	<0.001
U–meg/Cr + U–α$_1$-MG/ Cr	−1.963 (−2.847, −1.080)	<0.001
U–meg/Cr + U–β$_2$-MG/ Cr	−1.905 (−2.782, −1.028)	<0.001
U–meg/Cr + U-NAG/Cr	−1.940 (−2.829, −1.050)	<0.001

We compared the eGFR slopes of the higher megalin/Cr group with those of the lower megalin/Cr group. The participants in the higher megalin/Cr group had both a higher urinary A-megalin/Cr (≥222.52 pmol/g Cr) and higher urinary C-megalin/Cr (≥0.62 pmol/g Cr). The lower megalin/Cr group included all participants except those in the higher megalin/Cr group. The same analysis was conducted as described in the footnote to Table 2. U–meg, urinary megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U–α$_1$-MG, urinary α$_1$-microglobulin; U–β$_2$-MG, urinary β$_2$-microglobulin; U-NAG, urinary *N*-acetyl-β-D-glucosaminidase.

Cr group in model 1. Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend. The eGFR slope was −0.574 (95 % CI −1.122, −0.027) ml/min/1.73 m^2/year steeper in the higher C-megalin/Cr group than in the lower C-megalin/Cr group in model 1. In models adjusted with other urinary biomarkers, the slopes also showed a similar trend. These results indicate that urinary A- and C-megalin/Cr are likely to be prognostic biomarkers for DKD progression independent of other urinary biomarkers.

3.5. Longitudinal analysis of the association of combined urinary megalin excretion with eGFR slopes

According to the calculated cutoff points, we also divided the participants into two groups, a higher megalin/Cr group and a lower megalin/Cr group. The participants in the higher megalin/Cr group had both a higher urinary A-megalin/Cr (≥222.52 pmol/g Cr) and higher urinary C-megalin/Cr (≥0.62 pmol/g Cr). The lower megalin/Cr group included all participants except those in the higher megalin/Cr group. The baseline clinical characteristics of each group are shown in Appendix G.1. Each eGFR slope analyzed from the generalized linear model is shown in Table 4, Appendix G.2, and Fig. 1. The eGFR slope was −1.888 (95 % CI −2.764, −1.011) ml/min/1.73 m^2/year steeper in the higher megalin/Cr group than in the lower megalin/Cr group in the crude model or model 1 (Table 4, Appendix G.2, and Fig. 1). In addition, the difference in the eGFR slope of the higher and lower megalin/Cr groups was greater than the differences between the higher and lower A-megalin groups and between the higher and lower C-megalin groups (*P* = 0.008). Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend (Table 4 and Appendix G.2).

The 188 patients were also categorized into higher or lower megalin/Cr groups according to the median urinary A- and C-megalin/Cr levels at baseline. The characteristics of these groups are shown in Appendix H.1. Each eGFR slope analyzed from the generalized linear model is shown in Appendix H.2. The eGFR slope was −1.152 (95 % CI −1.756, −0.549) ml/min/1.73 m^2/year steeper in the higher megalin/Cr group than in the lower megalin/Cr group in model 1. Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend. Collectively, these results indicate that combined evaluation of urinary A- and C-megalin/Cr is more effective to predict the DKD progression.

3.6. Cross-sectional analysis of the association of urinary A- or C-megalin excretion with other parameters

Appendix I.1 shows the correlations of urinary A- and C-megalin excretion with other urinary biomarkers and clinical parameters at

baseline in the 188 patients Urinary A-megalin/Cr was correlated with eGFR ($r = 0.26$, $P = 0.001$) and U-NAG/Cr ($r = 0.19$, $P = 0.01$). In addition, urinary C-megalin/Cr was correlated with U-ACR ($r = 0.37$, $P < 0.001$), U–α$_1$-MG/Cr ($r = 0.26$, $P < 0.001$), U–β$_2$-MG/Cr ($r = 0.20$, $P = 0.005$), and U-NAG/Cr ($r = 0.29$, $P < 0.001$). Correlations between urinary biomarkers and other clinical parameters, excluding urinary megalin, are also shown in Appendix I.2.

4. Discussion

In this study, we established a novel deoxidization method to measure urinary A-megalin. Furthermore, in longitudinal analysis, we demonstrated that the eGFR slope of patients with a higher urinary A-megalin level was steeper than that of patients with a lower urinary A-megalin level. Similar results were obtained with urinary C-megalin. In models adjusted with other urinary biomarkers (U-ACR, U–α$_1$-MG/Cr, U–β$_2$-MG/Cr, and U-NAG/Cr), the eGFR slopes also showed a similar trend. These results suggest that the levels of two forms of urinary megalin can be used as independent prognostic biomarkers in the rapid progression of DKD. Furthermore, the eGFR slope was more steeper in the higher megalin/Cr group with higher urinary A- and higher C-megalin/Cr than in the group comprising the remaining patients (lower megalin/Cr group). The difference in the eGFR slope between the higher and lower megalin groups was also greater than the differences between the higher and lower A-megalin groups and between the higher and lower C-megalin groups, suggesting that urinary A- and C-megalin/Cr may play a synergistic role as prognostic biomarkers of DKD progression.

In this study, we evaluated the eGFR slopes as the indicator of DKD progression. In many clinical studies, the endpoints used to indicate DKD progression include an estimated glomerular filtration rate (eGFR) below 15 ml/min/1.73m^2, introduction of dialysis, renal death, doubling of the serum creatinine level, and a 40 % decline in the eGFR.[20,21] However, these endpoints require a large number of cases and a long observation period,[22] and it was not possible to analyze these endpoints in this study. On the other hand, albuminuria is another widely known indicator of DKD progression.[1,3] However, many cases have been reported of a decreased eGFR without an increase in albuminuria.[4,5] There are also reports of an increased risk of cardiovascular disease even in these normoalbuminuric patients.[35] For this reason, one method that has become accepted in recent years for predicting the risk of future end-stage renal failure involves analysis of the annual eGFR change rate.[23,30,36] Previous modeling analyses of CKD, including DKD, demonstrated that maintenance of the eGFR slope by $\geq −0.75$ ml/min/1.73 m^2/year over 3 years predicts a clinically relevant delay in progression with at least 96 % probability.[24,25] In our study, the groups with either higher urinary A-megalin/Cr or higher C-megalin/Cr or the group with both higher A- and higher C-megalin/Cr had eGFR slopes steeper than about −0.75 ml/min/1.73m^2/year, respectively, than the groups with lower values.

The mechanism underlying the excretion of urinary A-megalin has not been completely elucidated. However, we previously reported that the urinary excretion of A-megalin is decreased in children with oculocerebro-renal syndrome of Lowe (*OCRL*) gene mutations.[19] OCRL-1 is localized to clathrin-coated pits, early endosomes, and recycling endosomes in PTECs and has been implicated in endosome trafficking.[37] In Lowe syndrome and Dent-2 disease with *OCRL* gene mutations, OCRL-1–mediated megalin trafficking to the cell surface is impaired. As a result of the aberrant accumulation of actin at the endosomal membrane, megalin is retained in endosomes and missorted to lysosomes instead of being recycled to the brush border *via* recycling endosomes.[19] Subsequent dislocation of megalin at the cell surface may result in decreased urinary A-megalin excretion. However, in this study, the higher A-megalin/Cr group had a steeper eGFR slope than the lower A-megalin/Cr group. We hypothesize that endocytosis-related metabolic activation in proximal tubules would lead to upregulation of

intracellular recycling and extracellular cleavage of megalin.

We previously established that urinary C-megalin was elevated in DKD patients compared with normal controls.[17] In DKD patients, urinary C-megalin levels were also associated with serum vitamin D3 levels.[38] The clinical usefulness of urinary C-megalin is not limited to DKD because it is also associated with the severity of IgA nephropathy[39] and pediatric renal scarring,[40] reflecting the metabolic load to residual nephrons in these diseases. Thus, cross-sectional analyses have revealed that urinary megalin is related to various kidney diseases. In CKD patients, urinary C-megalin/Cr is associated with urinary iron and 8-hydroxydeoxyguanosine, an oxidative stress marker.[41] Moreover, we reported that urinary C-megalin is excreted *via* exosomes, and its urinary excretion is positively related to the increase in urinary albumin excretion.[18] In addition, cultured immortalized rat proximal tubule cells exhibited an increase in the excretion of exosomes with C-megalin from multivesicular bodies upon treatment with albumin and a further increase upon treatment with advanced glycation end product-modified albumin, both of which are endocytic ligands of megalin. These results indicate that increased urinary C-megalin excretion may be due to megalin-mediated quantitative or qualitative protein metabolic overload to the *endo*-lysosomal system of PTECs, triggering the development of DKD.[18] We show a schematic representation of the mechanisms and significance of urinary megalin excretion in Fig. 2.

In this study, cross-sectional analysis revealed a significant correlation between the baseline levels of urinary A-megalin and eGFR in our 188 patients with type 2 diabetes. However, there were no significant correlations of the baseline levels of urinary A-megalin with albumin, α_1-MG, β_2-MG, or NAG. The baseline levels of urinary C-megalin were significantly correlated with other urinary biomarkers, as we reported previously.[17] Although some studies reported urinary megalin without distinguishing between A-megalin and C-megalin,[38,42] our results suggest that A- and C-megalin are excreted in urine *via* different mechanisms. Furthermore, our study showed that the eGFR slope of the group with higher urinary A- and C-megalin/Cr was steeper than those of the groups with higher urinary A- or C-megalin, as shown in Fig. 1. Therefore, it might be important to measure urinary A-megalin and C-megalin

independently to detect the risk of a more rapid progression of DKD.

As mentioned above, none of our study patients were treated with SGLT2 inhibitors at baseline or during the follow-up period. Because SGLT2 is specifically located in proximal tubules, the inhibitors may also affect the function of other molecules expressed in the tubules. Indeed, β_2-MG, an endocytic ligand of megalin, was reported to be increased in the urine of type 2 diabetes patients who were administered ipragliflozin, an SGLT2 inhibitor.[43] In addition, luseogliflozin was found to ameliorate renal pathology but increase proteinuria in a rat model of diabetic nephropathy.[44] Indeed, SGLT2 inhibitors may alter the endocytic function of megalin.[45] Therefore, we may need to carefully evaluate urinary biomarkers in patients treated with SGLT2 inhibitors. Furthermore, use of SGLT2 inhibitors led to an initial drop in eGFR but eventually ameliorated the eGFR slope in type 2 diabetes patients.[32] Thus, in the present study, which investigated the eGFR slope in a rather short period, we analyzed the clinical data of patients who were not treated with SGLT2 inhibitors. However, in an ongoing trial, we are investigating the relationship between the SGLT2 inhibitor-induced changes in eGFR and urinary megalin excretion (UMIN000023902).

There are some limitations to this study. First, this was an exploratory retrospective study of a small cohort from a single hospital. In this cohort, many patients were in a relatively early stage of CKD. Moreover, the duration of the study was relatively short, and no patients progressed to end-stage renal disease and required renal replacement therapy. To solve these limitations, we plan to use a prospective, large, multicenter, and longer-term cohort including patients with a lower eGFR to clarify the usefulness of urinary megalin in predicting DKD progression using an outcome defined as progression to end-stage renal disease. Second, we could not analyze the cutoff point of urinary megalin with a validation cohort but used a development cohort. Furthermore, this cohort was retrospectively analyzed using urine samples collected between 2007 and 2011 in order to analyze clinical data from patients who were not taking SGLT2 inhibitors and to exclude the effects of these drugs on urinary megalin excretion and eGFR decline. Finally, ELISA kits to measure urinary A- and C- megalin are currently being developed. Therefore, they are not yet widely available. In addition, the limitation
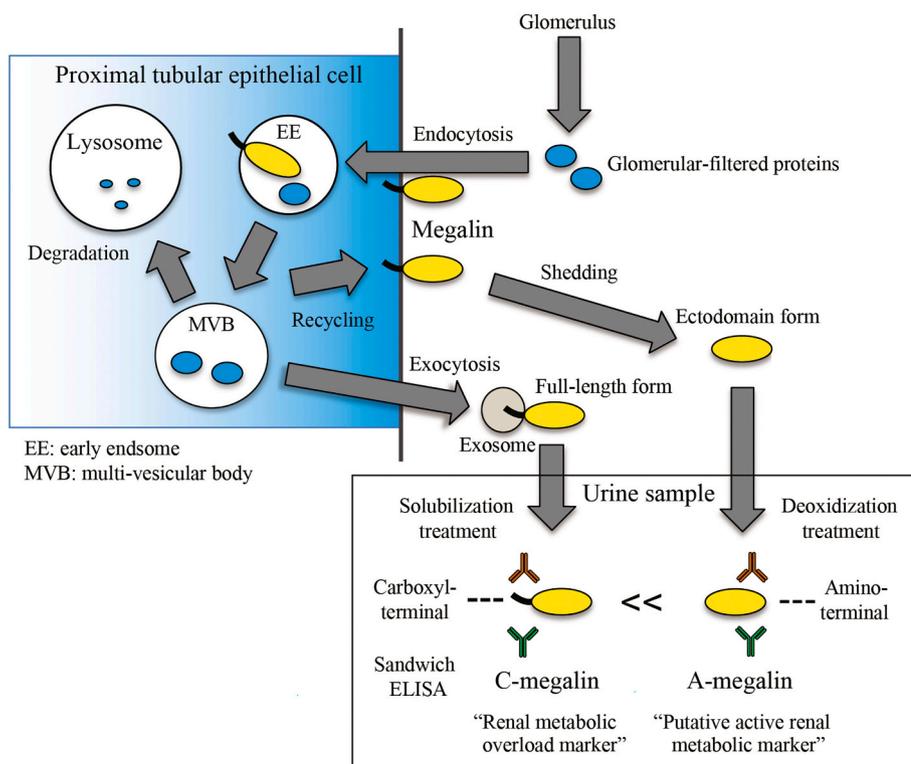
Fig. 2. Schematic representation of the mechanisms and significance of urinary megalin excretion.

Megalin endocytoses diverse glomerular-filtered substances including proteins into proximal tubular epithelial cells (PTECs). Endocytosed proteins are transported *via* endosomes to lysosomes for degradation. Megalin is recycled to the cell surface and shed into urine as an ectodomain form that is mainly measured by amino-terminal sandwich ELISA as "A-megalin", a putative active renal metabolic marker. In addition, megalin is exocytosed from multivesicular bodies into urine as a full-length form that is measured by carboxyl-terminal sandwich ELISA as "C-megalin", a marker of protein metabolic overload to the *endo*-lysosomal system of PTECs. The two forms of urinary megalin excretion may play discriminative and synergistic roles as biomarkers of diabetic kidney disease.

of this assay for A-megalin is its limited quantitative range. The detection sensitivity of this assay ranges from 3 to 3000 pmol/l.[17]

5. Conclusion

Urinary A-megalin was measured by a novel and simpler method than the one that we reported previously. Urinary A-megalin and C-megalin could be novel biomarkers that predict the eGFR decline in type 2 diabetes patients independently of other urinary biomarkers, including albumin, α_1-MG, β_2-MG, and NAG. Combined measurement of urinary A- and C-megalin/Cr is expected to serve as a more powerful predictor of DKD progression.

Declaration of competing interest

A.S. and S.G. received research grants from Denka Co., Ltd. The other authors declare that they have no competing interests.

CRediT authorship contribution statement

T.I., M.H., R.K., Y.S., I.N., and A.S. were responsible for the conception and design of the study. T.I. was the chief investigator and was responsible for data analysis. M.H., K.K., S.G., H.K., T.T., and N.K. were responsible for data analysis. T.I., M.H., Y.S., and A.S. were responsible for data acquisition. M.N., S.I., and S.O. were responsible for data measurement. T.I., M.H., H.K., T.T., N.K., and A.S. were responsible for data interpretation. T.I. and M.H. were responsible for drafting the manuscript. All authors contributed to the writing of the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2022.108312.

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