Original Paper

Urinary Iron Excretion is Associated with Urinary Full-Length Megalin and Renal Oxidative Stress in Chronic Kidney Disease

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Key Words

Urinary iron • Megalin • CKD • Oxidative stress

Abstract

Background/Aims: Megalin mediates the uptake of glomerular-filtered iron in the proximal tubules. Urinary full length megalin (C-megalin) excretion has been found to be increased in association with megalin-mediated metabolic load to the endo-lysosomal system in proximal tubular epithelial cells (PTECs) of residual nephrons. In the present study, we investigated the association between urinary iron and C-megalin in chronic kidney disease (CKD) patients, and the possible harmful effect of iron in renal tubules. **Methods:** Urinary levels of iron and C-megalin were measured in 63 CKD patients using automatic absorption spectrometry and a recently-established sandwich ELISA, respectively. **Results:** Although both urinary C-megalin and urinary total protein levels were correlated with urinary iron (C-megalin: $p = 0.574$, $p < 0.001$; total protein: $p = 0.500$, $p < 0.001$, respectively), urinary C-megalin alone emerged as an independent factor positively associated with urinary iron ($\beta = 0.520$, $p < 0.001$) ($R^2 = 0.75$, $p < 0.001$). Furthermore, urinary iron was significantly and positively associated with urinary 8-hydroxydeoxyguanosine, an oxidative stress marker, while no association with other markers of renal tubular injury, i.e., β_2 -microglobulin and N-acetyl- β -D-glucosaminidase, was noted. **Conclusions:** Our findings suggest that renal iron handling may be associated with megalin-mediated endo-lysosomal metabolic load in PTECs of residual nephrons and oxidative stress in renal tubules.

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Introduction

Iron, an essential component of hemoglobin, plays important roles in preservation of life [1, 2], with a daily dietary requirement of approximately 8 mg recommended for adult men and 18 mg for premenopausal women with menstrual iron loss [3]. Approximately 1–2 mg/day of iron is absorbed at the brush border of duodenal enterocytes. Circulating iron, mostly bound by its carrier proteins, enters the renal tubular lumen *via* the glomerulus, even under physiological conditions [4, 5]. Once filtered through the glomerulus, iron is nearly completely reabsorbed by endocytosis in the proximal and distal tubules [6], either through a transferrin-specific pathway, specifically, the transferrin receptor protein 1 pathway, or non-specific receptors such as megalin and cubilin [7, 8] in the form of non-transferrin-bound iron (NTBI) [6, 9]. However, iron is ultimately excreted in urine, which has been reported to include 62.4 ± 4.1 µg/g creatinine (Cr) in healthy subjects [10]. Free reactive iron, unbound by its carrier proteins, may mediate oxidative stress in the renal tubules [6, 11].

Megalin is a large (~600 kDa) glycoprotein that belongs to the low-density lipoprotein receptor family [12, 13] and highly expressed on the apical membrane of proximal tubular epithelial cells (PTECs) [14]. It plays a physiologically important role in the endocytic reabsorption of glomerular-filtered substances, such as albumin and low-molecular weight proteins including non-transferrin iron-carrier proteins in PTECs [6, 14]. Glomerular-filtered low-molecular weight proteins, such as α_1 -microglobulin [15], β_2 -microglobulin [16], and liver-type fatty acid binding protein [17], are clinical markers for the endocytic function of megalin. In addition, megalin mediates the uptake of nephrotoxic substances by PTECs, leading to development of CKD [18] and AKI [19]. Particularly, in a high-fat-induced obesity-related diabetes mouse model, megalin-mediated (auto) lysosomal dysfunction in PTECs was found to be primarily involved in development of tubuloglomerular alterations [18].

Increased urinary excretion of megalin, as evaluated by gel-based liquid chromatography-mass spectrometry, has been reported in patients with type 1 diabetes [20]. Recently, sandwich enzyme-linked immunosorbent assays (ELISAs) have been developed that are able to more precisely quantify ectodomain (A-megalin) and full-length (C-megalin) forms of megalin using monoclonal antibodies against the amino- and carboxyl-terminal of megalin, respectively [21]. Using these assays, urinary C-megalin/Cr levels were found to be elevated even in the normoalbuminuric stage of type 2 diabetes and increased further in conjunction with progression of diabetic nephropathy in those patients [21]. In addition, urinary C-megalin/Cr was reportedly associated with the severity of IgA nephropathy [22] and pediatric renal scarring [23]. Urinary C-megalin/Cr was also found to be increased *via* exocytosis in association with megalin-mediated quantitative or qualitative protein metabolic load to the endo-lysosomal system of PTECs in residual functional nephrons [24]. Although it has been proposed that urinary A-megalin excretion might be increased by accelerated intracellular recycling [25] and regulated intramembrane proteolysis of megalin [26], its clinical relevance remains to be determined.

We speculated that megalin-mediated handling of non-transferrin iron carrier proteins may be associated with development of CKD. Thus, urinary analysis of megalin and its endocytic ligands may be useful for clinical evaluation of the pathologic role of renal iron metabolism in CKD patients. Hence, the aims of the present study were to examine whether urinary iron excretion is associated with the level of urinary megalin or markers including megalin ligands, such as β_2 -microglobulin, in CKD patients, and also whether urinary iron level is related to pathologic oxidative stress using urinary 8-hydroxydeoxyguanosine (8-OHdG) as a marker [27–29].

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of Osaka City University Graduate School of Medicine (approval #603366). All study participants provided written informed consent, both for collection of blood and urine samples, and for examination of clinical records relevant to the study. The research was conducted in accordance with the Declaration of Helsinki.

Subjects

CKD was defined according to the criteria proposed by the Kidney Disease: Improving Global Outcomes organization [30]. CKD patients were regularly followed by nephrologists at the Department of Nephrology at Osaka City University Hospital, from which 63 patients were enrolled in March and April 2016. The etiology of the CKD patients included hypertensive nephrosclerosis (n=15), diabetic nephropathy (n=15), IgA nephropathy (n=6), membranous nephropathy (n=6), minimal change nephrotic syndrome (n=3), anti-neutrophil cytoplasmic antibody (MPO-ANCA)-associated glomerulonephritis (n=3), autosomal dominant polycystic kidney disease (n=2), membrano-proliferative nephropathy (n=2), focal and segmental glomerulosclerosis (n=2), and unknown cause (n=9). Patients undergoing treatment with oral drugs or supplements containing iron, including iron-containing phosphate binders, with malignancy, or a clinically overt infection were excluded. Thirteen of the 63 CKD patients were undergoing treatment with ESA.

Measurements

Spot blood and urine samples were collected from all subjects in the morning after overnight fasting. The urine samples were kept on ice for 1 h and then centrifuged at $630 \times g$ for 10 min, as previously described [31]. All laboratory measurements were performed using routine assays with automated methods [32]. Estimated glomerular filtration rate (eGFR) was calculated using the new Japanese coefficient for the abbreviated Modification of Diet in Renal Disease Study equation, including a correction factor of 0.739 for women [33]. Transferrin saturation (TSAT), a more sensitive biomarker of iron status than serum iron or total iron binding capacity, and which corresponds to circulating iron, was determined by multiplying the ratio of serum iron and total iron binding capacity by 100 [34]. Serum calcium was corrected based on serum albumin and determined as corrected calcium, as previously described [32]. Serum intact parathyroid hormone (PTH) was measured using a second-generation Elecsys PTH IRMA assay (Roche Diagnostics, Mannheim, Germany), as previously reported [32].

Urinary iron was measured by automatic absorption spectrometry, as previously reported [35, 36], using a Varian Spectra AA 220FS atomic absorption spectrophotometer (Palo Alto, CA). Quantification of urinary A-megalin and C-megalin was performed as previously described [21, 22]. In brief, 90- μ L urine samples were mixed with a 10- μ L solution (2 mol/L Tris-HCl, 0.2 mol/L EDTA, 10% Triton X-100, pH 8.0), and incubated for 1 min at room temperature for the C-megalin assay and for 3 h at 50°C for the A-megalin assay, to allow reactions between the capture monoclonal antibodies immobilized on the ELISA plates and the carboxy- and amino-terminal portions of megalin, respectively. An alkaline phosphatase-labeled tracer monoclonal antibody was then added to the plate and measurements were conducted using a chemiluminescent immunoassay detection system. The intra- and inter-assay coefficients of variation were less than 10%. Urinary concentrations of Cr, N-acetyl- β -D-glucosaminidase (NAG), and β_2 -microglobulin were measured using an automated instrument (7170S; Hitachi High-Technologies Corp., Tokyo, Japan) with CRE-S (Denka Seiken Co., Ltd., Gosen, Japan), N-assay L NAG NITTOBO (Nittobo Medical Co., Ltd., Tokyo, Japan), and BMG-Latex (Denka Seiken Co., Ltd., Gosen, Japan) kits, respectively, as previously described [21]. As a marker for oxidative stress in urine, urinary 8-OHdG was measured by ELISA (Japan Institute for the Control of Aging, NIKKEN SEIL, Co., Ltd., Shizuoka, Japan), as previously described [28]. The urinary concentration of each marker was normalized to that of Cr and expressed as g/g Cr (total protein), mg/g Cr (β_2 -microglobulin), μ g/g Cr (iron, transferrin, and 8-OHdG), pmol/g Cr (A-megalin and C-megalin), or U/g Cr (NAG).

Table 1. Clinical characteristics of the 63 CKD patients. Data are expressed as the mean $\pm$ SD. Medians [interquartile range] are shown for variables with skewed distributions. eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; TSAT, transferrin saturation; HbA1c, hemoglobin A1c; PTH, parathyroid hormone; Cr, creatinine; NAG, N-acetyl- β -D-glucosaminidase; 8-OHdG, 8-hydroxydeoxyguanosine

Clinical variables	n = 63
Male/female	49/14
Age (years)	65.2 $\pm$ 14.1
eGFR (mL/min/1.73 m ²)	26.6 $\pm$ 14.5
Creatinine (mg/dL)	2.2 [1.2–3.4]
BUN (mg/dL)	29 [13–49]
Hemoglobin (g/dL)	12.4 $\pm$ 1.75
Iron (μ g/dL)	89.4 $\pm$ 34.7
Ferritin (ng/mL)	104 [52.6–197]
TSAT (%)	31.9 [21.5–42.5]
Erythropoietin (mIU/mL)	12.8 [8.1–19]
Fasting plasma glucose (mg/dL)	107 [91–129]
HbA1c (%)	5.60 [5.38–6.13]
Corrected calcium (mg/dL)	9.4 [9.0–9.7]
Phosphate (mg/dL)	3.7 [3.2–4.4]
Intact PTH (pg/mL)	138 [1.2–3.4]
Total protein (g/dL)	6.61 $\pm$ 0.75
Albumin (g/dL)	3.57 $\pm$ 0.55
Urinary total protein (g/g Cr)	2.60 [1.20–5.40]
Urinary transferrin (μ g/g Cr)	107 [47.8–203]
Urinary β_2 -microglobulin (mg/g Cr)	15.4 [3.46–130]
Urinary NAG (IU/g Cr)	16.0 [9.10–20.5]
Urinary 8-OHdG (μ g/g Cr)	90.9 [75.9–108]
Urinary iron (μ g/g Cr)	109 [69.3–166]
Urinary A-megalin (pmol/g Cr)	35.2 [23.5–55.0]
Urinary C-megalin (pmol/g Cr)	0.90 [0.50–1.30]

Statistical analysis

Continuous variables distributed normally are expressed as the mean $\pm$ standard deviation (SD). The median [interquartile range] was used for continuous variables with a skewed distribution. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) or JMP software version 10 (SAS Institute, Inc., Cary, NC, USA). Correlations between urinary iron and clinical data were examined by Pearson's and Spearman's analyses for parametric and nonparametric data, respectively. Multiple regression analyses were performed to assess independent associations among urinary iron, C-megalin, and 8-OHdG. P-values <0.05 were considered to be statistically significant.

Drug dosage

The authors and the publisher have exerted every effort to ensure that drug selection and dosages set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information related to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any changes in indications and dosage, and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

Results

Clinical characteristics of CKD patients

The clinical characteristics of the 63 CKD patients enrolled in this study are shown in Table 1. Data are presented as the mean $\pm$ SD or medians [interquartile range]. The mean $\pm$ SD of eGFR was 26.6 $\pm$ 14.5 mL/min/1.73 m². The serum levels of iron, ferritin, and TSAT were 89.4 $\pm$ 34.7 μ g/mL, 104 [52.6–197] ng/mL, and 31.9% [21.5–42.5%], respectively, which were nearly within their respective normal ranges. Urinary iron was 109 [69.3–166] μ g/g Cr, which was higher than the value reported for healthy subjects (62.4 $\pm$ 4.10 μ g/g Cr, n=97) [10]. Urinary C-megalin was 0.90 [0.50–1.30] pmol/g Cr, which was higher than that reported for healthy subjects (0.15 pmol/g Cr, n=160), though similar to that for type 2 diabetic patients with macroalbuminuria (1.32 pmol/g Cr, n=13) [21]. Urinary A-megalin

Table 2. Correlation of urinary iron levels with clinical factors in 63 CKD patients. Data include simple correlation coefficients (ρ -value) and level of significance (p -value) (bold if $p < 0.05$). eGFR, estimated glomerular filtration; BUN, blood urea nitrogen; TSAT, transferrin saturation; HbA1c, hemoglobin A1c; PTH, parathyroid hormone; Cr, creatinine; NAG, N-acetyl- β -D-glucosaminidase; 8-OHdG, 8-hydroxydeoxyguanosine

Clinical variables	Correlation with urinary iron levels	
	ρ	p
Age (years)	0.066	0.605
eGFR (mL/min/1.73 m ²)	-0.074	0.563
Creatinine (mg/dL)	0.054	0.676
BUN (mg/dL)	0.025	0.847
Hemoglobin (g/dL)	-0.134	0.286
Iron (μ g/dL)	0.156	0.218
TSAT (%)	0.358	0.004
Ferritin (ng/mL)	0.185	0.146
Fasting plasma glucose (mg/dL)	-0.161	0.209
HbA1c (%)	0.025	0.863
Phosphate (mg/dL)	-0.003	0.464
Corrected calcium (mg/dL)	0.139	0.285
Intact PTH (pg/mL)	0.096	0.464
Total protein (g/dL)	-0.473	<0.001
Albumin (g/dL)	-0.507	<0.001
Urinary total protein (g/g Cr)	0.500	<0.001
Urinary transferrin (μ g/g Cr)	0.474	<0.001
Urinary β_2 -microglobulin (mg/g Cr)	0.497	<0.001
Urinary NAG (IU/g Cr)	0.440	<0.001
Urinary 8-OHdG (μ g/g Cr)	0.260	0.048
Urinary A-megalin (pmol/g Cr)	-0.215	0.091
Urinary C-megalin (pmol/g Cr)	0.574	<0.001

was 35.3 [23.5–55.0] pmol/g Cr, which was similar to that for type 2 diabetic patients with macroalbuminuria (63 pmol/g Cr, $n=13$) [21].

Urinary iron, C-megalin, A-megalin, and 8-OHdG levels were not significantly different between patients with diabetic nephropathy and those with non-diabetic renal disease (urinary iron: 127 [76.0–316] vs. 101 [66.9–166] μ g/g Cr, $p = 0.475$; urinary C-megalin: 0.64 [0.44–1.99] vs. 0.85 [0.51–1.14] pmol/g Cr, $p = 0.273$; urinary A-megalin: 35.0 [15.8–45.9] vs. 36.7 [25.5–56.3] pmol/g Cr, $p = 0.378$, and urinary 8OHdG: 83.3 [73.0–103] vs. 91.5 [75.6–112] μ g/g Cr, $p = 0.559$, respectively).

Correlations between urinary iron levels and various clinical parameters in CKD patients

The correlations between urinary iron levels and various clinical parameters were examined in CKD patients using simple regression analyses (Table 2). While the level of urinary iron exhibited significant correlations with urinary total protein ($\rho = 0.500$, $p < 0.001$) (Fig. 1A), urinary transferrin ($\rho = 0.474$, $p < 0.001$), urinary β_2 -microglobulin ($\rho = 0.497$, $p < 0.001$), and urinary NAG ($\rho = 0.440$, $p < 0.001$), it was not correlated with eGFR, serum Cr, or urea nitrogen. Furthermore, a preferential correlation was found between urinary iron and urinary C-megalin ($\rho = 0.574$, $p < 0.001$) (Fig. 1B), but not between urinary iron and urinary A-megalin ($\rho = -0.215$, $p = 0.091$) (Fig. 1C). Of importance, urinary iron had a significant positive correlation with urinary 8-OHdG ($\rho = 0.260$, $p = 0.048$) (Fig. 2), a marker of oxidative stress [27–29]. Among the serum parameters examined, urinary iron showed a significant positive correlation with TSAT ($\rho = 0.358$, $p = 0.004$).

Correlations between urinary C-megalin level and various clinical parameters

We next investigated parameters associated with urinary C-megalin. While urinary β_2 -microglobulin, urinary NAG, urinary total protein, urinary transferrin, eGFR, serum Cr, urea nitrogen, hemoglobin, total protein, and albumin were significantly correlated with urinary C-megalin, urinary A-megalin was not correlated with those parameters (Table 3, Fig. 3).

Fig. 1. Correlations between urinary protein, C-megalin, A-megalin, and urinary iron excretion. Urinary protein was positively correlated with urinary iron (A). Urinary C-megalin was positively correlated with urinary iron (B), while urinary A-megalin was not (C). Simple correlation coefficients and level of significance (p-value) are presented.

Fig. 2. Correlations between urinary total protein and urinary C-megalin. Urinary total protein was significantly and positively correlated with C-megalin. Simple correlation coefficients and level of significance (p-value) are presented.

Table 3. Correlation between the clinical factors and urinary C-megalin levels in 63 CKD patients (simple linear regression analysis). Data include the simple correlation coefficients (ρ -value) and the levels of significance (p-value) (bolded if $p < 0.05$). eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; TIBC, total iron binding capacity; HbA1c, hemoglobin A1c; Cr, creatinine; NAG, N-acetyl- β -D-glucosaminidase

Clinical Variables	Correlation with urinary C-megalin levels	
	ρ	p
Age (years)	0.093	0.464
eGFR (mL/min/1.73 m ²)	-0.293	0.022
Creatinine (mg/dL)	0.278	0.027
BUN (mg/dL)	0.323	0.010
Hemoglobin (g/dL)	-0.328	0.009
Iron (µg/dL)	-0.103	0.425
TSAT (%)	0.068	0.600
Ferritin (mg/dL)	0.037	0.769
Fasting plasma glucose (mg/dL)	-0.241	0.057
HbA1c (%)	-0.153	0.287
Phosphate (mg/dL)	0.158	0.235
Corrected calcium (mg/dL)	0.048	0.713
Total protein (g/dL)	-0.367	0.003
Albumin (g/dL)	-0.405	0.001
Urinary total protein (g/g Cr)	0.528	<0.001
Urinary transferrin (µg/g Cr)	0.266	0.042
Urinary β_2 -microglobulin (mg/g Cr)	0.513	<0.001
Urinary NAG (IU/g Cr)	0.364	0.003
Urinary A-megalin (pmol/g Cr)	0.347	0.005

Fig. 3. Correlations between urinary iron and urinary 8-OHdG. Urinary iron was significantly and positively correlated with urinary 8-OHdG, an oxidative stress marker. Simple correlation coefficients and level of significance (p-value) are presented. 8-OHdG, 8-hydroxydeoxyguanosine

Table 4. Multivariate analysis to elucidate factors associated with urinary iron levels and clinical parameters in 63 CKD patients. Data are shown as standard regression coefficients (β -value) and levels of significance (p-value) (bold if $p < 0.05$). R^2 = multiple coefficient of determination. Ur, urinary; eGFR, estimated glomerular filtration rate; TP, total protein; TSAT, transferrin saturation; 8-OHdG, 8-hydroxydeoxyguanosine

Term	Model 1		Model 2 (Model 1 + C-megalin)		Model 3 (Model 1 + A-megalin)	
	β	p	β	p	β	p
Age	0.081	0.381	0.054	0.488	0.092	0.324
Sex	0.015	0.867	0.144	0.078	0.007	0.941
eGFR	0.129	0.145	0.126	0.094	0.159	0.095
TSAT	0.236	0.012	0.300	<0.001	0.224	0.018
Ur TP	0.563	<0.001	0.159	0.179	0.559	<0.001
Ur 8-OHdG	0.372	<0.001	0.296	<0.001	0.379	<0.001
Ur C-megalin	-	-	0.520	<0.001	-	-
Ur A-megalin	-	-	-	-	-0.084	0.320
R^2	0.65 ($p < 0.001$)		0.75 ($p < 0.001$)		0.65 ($p < 0.001$)	

Table 5. Multivariate analysis to elucidate factors associated with urinary 8-OHdG levels and clinical parameters in 63 CKD patients. Data are shown as standard regression coefficients (β -value) and level of significance (p-value) (bold if $p < 0.05$). R^2 = multiple coefficient of determination. 8-OHdG, 8-hydroxydeoxyguanosine; Ur, urinary; eGFR, estimated glomerular filtration rate; MEG, megalin; TP, total protein; Tf, transferrin; β_2 MG, β_2 -microglobulin; NAG, N-acetyl- β -D-glucosaminidase

Term	Model 1		Model 2		Model 3		Model 4		Model 5	
	β	p	β	p	β	p	β	p	β	p
Age	0.264	0.019	0.237	0.037	0.246	0.031	0.268	0.022	0.272	0.018
Sex	-0.088	0.428	-0.024	0.829	-0.032	0.771	-0.061	0.584	-0.061	0.588
eGFR	0.026	0.815	0.007	0.953	0.047	0.668	0.059	0.627	0.068	0.542
Ur Iron	0.672	<0.001	0.652	<0.001	0.640	<0.001	0.517	<0.001	0.504	<0.001
Ur C-MEG	-0.240	0.137	-	-	-	-	-	-	-	-
Ur TP	-	-	-0.241	0.113	-	-	-	-	-	-
Ur Tf	-	-	-	-	-0.205	0.177	-	-	-	-
Ur β_2 MG	-	-	-	-	-	-	-0.027	0.858	-	-
Ur NAG	-	-	-	-	-	-	-	-	-0.008	0.952
R^2	0.40 ($p < 0.001$)		0.40 ($p < 0.001$)		0.40 ($p < 0.001$)		0.38 ($p = 0.001$)		0.37 ($p = 0.001$)	

Multivariate analyses to elucidate parameters associated with urinary iron levels

We performed multivariate analyses to further identify urinary parameters independently associated with urinary iron (Table 4), and found that urinary total protein ($\beta = 0.563$, $p < 0.001$) and urinary 8-OHdG ($\beta = 0.372$, $p < 0.001$) were significant factors positively associated with urinary iron excretion ($R^2 = 0.65$, $p < 0.001$), after adjustment for age, gender, eGFR, and TSAT (Table 4, Model 1). In Model 2, which added urinary C-megalin as an independent variable to Model 1, urinary C-megalin ($\beta = 0.520$, $p < 0.001$), but not

urinary total protein ($\beta = 0.159$, $p = 0.179$), emerged as an independent and significant factor positively associated with urinary iron excretion ($R^2 = 0.75$, $p < 0.001$). However, urinary A-megalin was not associated with urinary iron excretion (Table 4, Model 3).

Clinical significance of increased urinary iron excretion

Urinary iron was significantly and positively associated with urinary 8-OHdG (Table 4). To examine whether urinary iron is directly involved in increased oxidative stress in the renal tubules or whether other parameters are also associated with oxidative stress, multivariate analyses were simultaneously performed, including urinary iron levels and other parameters. As shown in Table 5, urinary 8-OHdG was significantly associated with urinary iron, but not with other markers including urinary C-megalin ($\beta = -0.240$, $p = 0.137$), urinary total protein ($\beta = -0.241$, $p = 0.113$), urinary transferrin ($\beta = -0.205$, $p = 0.117$), urinary β_2 -microglobulin ($\beta = -0.027$, $p = 0.858$), or urinary NAG ($\beta = -0.008$, $p = 0.952$).

Discussion

The present study demonstrated that urinary iron excretion in CKD patients is significantly associated in a positive manner with urinary C-megalin, a marker of megalin-mediated metabolic load to the endo-lysosomal system of PTECs in residual functional nephrons, and with urinary 8-OHdG, a marker of oxidative stress [27-29]. The associations of urinary iron excretion with C-megalin and 8-OHdG were independent of other urinary markers. Urinary iron excretion is determined by glomerular filtration and proximal tubular uptake of iron-carrier complexes. Thus, it is likely that the process of renal iron handling may be associated with megalin-mediated metabolic load to PTECs and oxidative stress in the renal tubules [37, 38], possibly through intracellular and intraluminal accumulation of iron as a generator of oxidative stress in the renal tubules [29, 39].

We also confirmed that urinary total protein is significantly and positively associated with urinary C-megalin (Table 3). Such an association is quite reasonable, since increased glomerular filtration of proteins, which are taken up by PTECs *via* megalin, should then overload the cellular endo-lysosomal system, leading to increased urinary C-megalin/Cr excretion mediated by exocytosis [24]. In addition, increased glomerular filtration of pathologic proteins, such as fatty acid-bound [18] or advanced glycation end product-modified albumin [24], would also load the system and stimulate PTECs *via* megalin to synthesize chemokines and growth factors, including monocyte chemoattractant protein-1, RANTES (regulated upon activation, normal T cell expressed and secreted), and fractalkine, which recruit monocytes and T-cells, as well as interleukin-8, which attracts neutrophils [40-44], causing tubulointerstitial damage and nephron loss [45, 46]. Consequently, in patients in an advanced stage of CKD, protein reabsorption and the metabolic pathway in PTECs of remnant nephrons, both of which are mediated by megalin, are likely promoted, further increasing urinary C-megalin/Cr excretion [24].

In addition, we found that urinary iron as well as C-megalin were significantly elevated in patients with nephrotic proteinuria ($n=27$) as compared to those with non-nephrotic proteinuria ($n=36$) (urinary iron: 139 [99.9-253] vs. 83.2 [58.2-149] $\mu\text{g/g Cr}$, $p = 0.004$; urinary C-megalin: 1.08 [0.80-1.99] vs. 0.55 [0.36-0.95] pmol/g Cr , $p < 0.001$, respectively). Thus, increased glomerular protein filtration is not only associated with residual nephron metabolic load but also increased renal iron handling.

In contrast, urinary A-megalin levels were significantly lower in patients with nephrotic proteinuria ($n=27$) as compared to those with non-nephrotic proteinuria ($n=36$), (29.1 [18.0-51.9] vs 42.6 [28.9-65.4] pmol/g Cr , $p = 0.049$). These results were similar to those of a previous study that investigated urinary A-megalin in diabetic patients, in which urinary A-megalin levels were found to be increased in diabetic patients with normo- and microalbuminuria, but not in those with macroalbuminuria [21]. Although urinary A-megalin

excretion might be elevated by distinct mechanisms, as noted above [25, 26], the clinical importance of that increase remains to be investigated further.

In the present study, the median urinary iron level in CKD patients was 109 [69.3–166] µg/g Cr (Table 1), higher than that reported for healthy subjects (62.4 ± 4.1 µg/g Cr) [10]. Furthermore, the urinary iron levels in our patients were similar to those noted for patients with diabetic nephropathy (150 ± 166 µg/g Cr, $n = 10$) [47], and nephrotic patients with focal and segmental glomerular sclerosis (4 ± 1 µmol/L, $n = 7$) [48]. Therefore, our findings are considered to reflect the state of general CKD patients with proteinuria.

Our results indicate that urinary transferrin is significantly correlated in a positive manner with urinary iron ($\rho = 0.474$, $p < 0.001$). Since transferrin is the major iron-transporting protein in serum, glomerular leakage of transferrin-bound iron is likely increased in glomerular filtrate along with increased glomerular filtration of serum proteins. Circulating iron mainly binds tightly to transferrin through extremely high affinity iron-specific binding, which stabilizes iron so that it cannot participate in radical-generating reactions [49, 50]. However, previous reports have shown that serum iron also binds to other filterable iron-binding proteins, such as albumin, ceruloplasmin, lactoferrin, neutrophil gelatinase-associated lipocalin, and hepcidin, in the form of NTBI [6, 51, 52], even in patients without iron overload. NTBI has been suggested to be a potent generator of free reactive radicals, as it is not shielded by the protective carrier protein transferrin [53]. Therefore, the major form of iron in glomerular filtrate is considered to be transferrin-bound iron, which undergoes endocytosis by transferrin-specific pathways, that is, the transferrin receptor protein 1 pathway [5], whereas other iron-carrier proteins may be increased in the glomerular filtrate of CKD patients with enhanced glomerular protein filtration. Those non-transferrin iron-carrier proteins may be endocytosed preferentially by megalin or cubilin, and then accumulate in the endo-lysosomal system of PTECs [51, 52]. In addition, those proteins may be retained in the intraluminal space of renal tubules when megalin- or cubilin-mediated reabsorption in PTECs is impaired or surpassed by increased glomerular filtration. Thus, in CKD patients, it is likely that NTBI accumulates in both PTECs and the intraluminal space of tubules to generate free reactive radicals. Furthermore, since the binding of transferrin and other iron-carrier proteins with iron is decreased in conjunction with a reduction of pH below neutrality [51], it is possible that the progressive decrease of pH in the renal tubules in those patients might dissociate more iron from its carrier proteins. Indeed, as noted above, urinary iron levels are associated significantly with urinary 8-OHdG, a widely used marker of systemic and renal tubular oxidative stress [27, 54–56] (Table 2, Table 3). Since the association of urinary iron with urinary 8-OHdG was shown to be independent of other urinary markers, including urinary total protein, transferrin, β_2 -microglobulin, and NAG, it is strongly suggested that increased tubular iron directly causes free reactive radicals to increase oxidative stress in the renal tubules. Indeed, accumulating evidence has shown that iron and iron-containing molecules cause direct injury to renal tubular cells *in vitro* [7, 57] as well as *in vivo* in mice [7, 58].

The present study has a number of limitations. First, the number of subjects examined was relatively small, with a predominance of males, mainly because we enrolled the study subjects from patients consecutively treated at a single institution. It is also not clear whether the results obtained from the current study can be extended to other ethnicities. Additional studies with larger sample sizes including other ethnicities are needed to verify our results. Additionally, the cause of CKD in the present subjects was mainly glomerular diseases, although some were complicated with tubular damage. None of the subjects had biopsy-proven genuine tubulointerstitial nephritis. However, as shown in Table 2, we found an association of urinary iron with urinary β_2 -microglobulin, a marker of proximal tubular dysfunction that leads to decreased endocytic uptake of β_2 -microglobulin by megalin. Finally, we were unable to identify any potential roles of erythropoiesis-stimulating agents (ESA) in increased urinary iron excretion. Regulation of iron transport may be biased towards minimization of urinary iron loss to ensure adequate iron bioavailability for erythropoiesis,

regardless of systemic iron content. Indeed, in the present study, 13 of the 63 CKD patients were undergoing treatment with ESA. While we cannot exclude the potential action of ESA on urinary iron excretion, urinary iron excretion levels were not significantly different between subjects with and without ESA (134.3 ± 77.4 vs. $152 \pm 128 \mu\text{g/g Cr}$, $p = 0.758$). Further studies are needed to investigate the effects of ESA and iron supplementation, including iron-based phosphate binders, on urinary iron excretion.

This is the first report to investigate the association between urinary iron and urinary megalin forms in CKD patients.

Conclusion

We found that urinary C-megalin is an important factor associated with increased urinary iron excretion and has an independent association of increased urinary iron with urinary oxidative stress. Urinary C-megalin may be a promising novel biomarker that reflects protein metabolic load in residual nephrons and renal iron handling, and could be useful to predict future progression of CKD. Additional longitudinal studies on its clinical relevance will be required.

Disclosure Statement

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