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Urine markers of renal tubular injury in idiopathic membranous nephropathy: A cross sectional study

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ABSTRACT

Introduction: Idiopathic membranous nephropathy (IMN) is a primary glomerular disease and a major cause of adult nephrotic syndrome. Presently, little is known about the capabilities of the urine markers to reflect the severity of IMN. We aimed at establishing whether urinary *N*-acetyl-β-glucosaminidase (NAG), Retinol binding protein (RBP), Kidney injury molecule-1 (KIM-1) and Neutrophil gelatianse-associated lipocalin (NGAL) are related with renal parameters and the histological grades tubular injury.

Methods: The levels of urinary NAG, RBP, KIM-1 and NGAL were determined in 165 biopsy-proven patients and 64 healthy controls. Their levels were then compared between patients and healthy subjects, and between patients with and without nephrotic syndrome. Their linearity with renal parameters and associations with histological grades of renal tubular injury were also assessed.

Results: All biomarkers were significantly increased in patients ($p < .001$). However, no significant increase was observed between patients exhibiting moderate and severe grades tubular injury and those exhibiting mild histological grade. With exception of RBP, all biomarkers were higher in patients with nephrotic syndrome ($p < .001$) and significantly correlated with majority of renal parameters including proteinuria.

Conclusion: Our findings suggest that although urine markers of tubular injury are increased in IMN, they may not offer a reflection of histological grades.

1. Introduction

Idiopathic membranous nephropathy (IMN) is a subtype of mebranous nephropathy and a major cause of adult nephrotic syndrome [1]. Also known as primary membranous nephropathy, IMN is a glomerular disease whose pathogenesis is not yet fully clarified. It accounts for the biggest proportion of membranous nephropathy and is characterized by the subepithelial glomerular deposits whose composition is mainly immunoglobulin G and complement 3. In addition to glomerular injuries on which the severity of IMN is dependent, tubulointerstitial injury occurs and has recently been associated with its prognosis [2]. The role of renal tubular injury in the progression and prognosis of IMN has for so long been downplayed in spite of the existing body of evidence in other kidney diseases [3–6]. Despite the apparent role of renal tubular injury in the progression of renal diseases, there is scanty information regarding the applicability of the urine markers in IMN, an important cause of nephrotic syndrome among adults. It is unknown whether the urine markers are capable of reflecting renal tubular injuries and the severity of IMN. Unknown also is their potential to predict IMN's course, the knowledge of which is crucial for timely treatment required to reduce the risk of end stage renal disease (ESRD) and avoiding unnecessary exposure of patients to immunosuppression therapy. Presently, the clinical course of IMN is predicted using proteinuria [7,8] the onset of which lags behind kidney injury. Accordingly, new biomarkers capable of predicting the course of IMN before significant renal damage are needed.

N-acetyl-β-D-glucosaminidase (NAG) and retinol-binding protein (RBP) are the urinary markers of renal tubular damage in current clinical practice. Kidney injury molecule 1(KIM-1) and neutrophil gelatianse-associated lipocalin (NGAL) are relatively new markers which, like NAG and RBP, have hardly been studied in IMN.

Abbreviations: CKD, chronic kidney disease; Cys-C, cystatin C; FBS, fasting blood sugar; IMN, Idiopathic membranous nephropathy; KIM-1, kidney injury molecule-1; NAG, *N*-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatianse-associated lipocalin; PALB, prealbumin; RBP, retinol-binding protein; KDIGO, kidney diseases improving global outcomes

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2. Materials and methods

2.1. The diagnostic and inclusion criteria for patients and healthy subjects

The study was conducted at Shengjing hospital of China Medical University with 165 patients and 64 healthy subjects recruited from the nephrology and health screening departments respectively. The patients and healthy controls were matched for age and sex and recruited into the study starting from March 2015 to July 2017.

Only biopsy-proven IMN patients for whom the causes of secondary membranous nephropathy (SMN) had been excluded by routine screening were recruited. Infections such as HIV, Hepatitis B and malaria, malignances and drugs associated with SMN were ruled out among the patients. No patient had received either glucocorticoids or immunosuppression therapy at the time of the study.

Histologically, tubular injury was scored as < 25%, 25–50% and > 50% for mild, moderate and severe grades respectively as previously reported [9,10]. Out of the 165 patients, no patient was without tubular injury. The number of patients with mild, moderate and severe histological grades of renal tubular injury were 99, 61 and 5 respectively. After recruitment, patients were divided into 2 groups according to their concentration of proteinuria i.e. remission group (proteinuria < 350 mg/mmol creatinine) and the non-remission group (proteinuria > 350 mg/mmol creatinine) as specified in the 2012 kidney diseases improving global outcomes (KDIGO) clinical practice guidelines [11]. The study was approved by the Ethics Committee of Shengjing hospital of China medical university.

2.2. Collection of samples and basic information

The samples, demographic and clinical information were obtained from patients and healthy subjects who met the inclusion criteria. Early morning urine samples were collected using clean plastic containers. Whole blood samples were collected on the same day as urine using plain and EDTA anti-coagulated vaccutainers. Blood in the plain vaccutainers was allowed to clot thoroughly before spinning to obtain serum. Urine was centrifuged at 3000 rpm/min for 4 min to obtain supernatants. Both serum and urine supernatants were stored at -80 °C pending analysis.

2.3. Quantification of urinary N-acetyl-β-D-glucosaminidase, retinol binding protein, Neutrophil gelatianse-associated lipocalin , kidney injury molecule-1 and other clinical parameters

NAG, RBP, NGAL and all other biochemical investigations were measured by Architect C16000 analyzer (Abbott Labs.) while KIM-1 was measured with sandwich enzyme immunoassay kits (USCN Life Sciences, SEA785Hu). Hematological parameters were obtained from full blood count results measured by DxH 600 Hematology Analyzer (Beckman-Coulter). The urine concentration of NAG was determined kinetically with reagents purchased from Beijing Leadman Biochemical Co. Ltd., China while RBP was measured with immunoturbidimetric assay kits from the same supplier. NGAL was measured with latex-enhanced immunoturbidimetric assay kits from Nanjing Vazyme Medical Technology Co. Ltd., China. All procedures were performed in accordance with manufacturers' instructions.

2.4. Statistical analysis

Data analysis was performed using SPSS 19.0 statistical software package. Gender composition of patients and health subjects was compared using the chi-square test while the age, general clinical parameters and the urinary markers of tubular injury were compared using the *t*-test and Mann-Whitney *U* test as appropriate. Normality of the data was tested with Kolmogorov–Smirnov test while correlations were determined by Spearman's rank correlation coefficients. All tests were two-sided and the difference was statistically significant when P < .05.

Table 1
Comparison of demographic and clinical information of patients and health controls.

Parameters	IMN patients (*N* = 165)	Healthy controls (*N* = 64)	p-value
Gender (male/female)	109/56	42/22	1.000
Age (y)	49.04 ± 11.98	47.08 ± 5.66	0.210
Cr (μmol/l)	66.60 (55.85–78.55)	63.55 (58.87–73.22)	0.289
Urea (mmol/l)	6.18 ± 2.04	5.01 ± 1.40	< 0.001
Cys-C (mg/l)	1.22 (1.03–1.39)	0.83 (0.74–0.92)	< 0.001
Uric acid (μmol/l)	339.75 ± 77.61	313.62 ± 59.42	0.017
TP (g/l)	46.29 ± 9.06	73.96 ± 4.11	< 0.001
Alb (g/l)	25.99 ± 7.40	47.20 ± 2.46	< 0.001
Urine protein (g/l)	3.11 (1.38–5.81)	0.05 (0.02–0.07)	< 0.001
eGFR (ml/ min*1.73 m^2)	103.81 (96.13–113.43)	105.51 (98.13–113.43)	0.157
TC (mmol/l)	7.14 ± 2.91	4.85 ± 0.80	< 0.001
TG (mmol/l)	2.21 (1.43–2.81)	0.96(0.72–1.55)	< 0.001
WBC (10^9/l)	8.81 ± 3.23	6.19 ± 1.48	< 0.001
RBC (10^{12}/l)	4.20 ± 0.57	4.95 ± 0.48	< 0.001
HGB (g/l)	129.01 ± 18.29	148.09 ± 16.11	< 0.001
PLT (10^9/l)	230.76 ± 62.09	242.48 ± 46.72	0.173
ALT (U/l)	16.00 (13.00–25.00)	18.00 (13.00–28.50)	0.304
AST (U/l)	15.00 (12.00–18.00)	15.00 (12.25–19.75)	0.764
PALB (g/l)	0.28 ± 0.08	0.32 ± 0.06	< 0.001
A/G	1.28 ± 0.4	1.81 ± 0.32	< 0.001

eGFR: estimated glomerular filtration, ALT: alanine transferase, AST: aspartate transferase, FBS: fasting blood sugar, PLT: platelet count, Cys−C: cystatin C, TC: total cholesterol, TG: triglycerides,WBC: white blood cell count total, TP: total protein Albumin(ALB), PALB: prealbumin, A/G: albumin-globulin ratio, RBC: red blood cell count HGB: hemoglobin, Cr: creatinine, IMN: idiopathic membranous nephropathy.

3. Results

All results of urine investigations including NAG, NGAL, KIM-1 and RBP were standardized with urine creatinine and reported.

3.1. Comparison of demographic and clinical data between patients and healthy subjects

Demographic and clinical characteristics of patients and healthy subjects were compared as shown in Table 1. Between patients and healthy controls, the differences in age, sex ratio, serum creatinine, estimated glomerular filtration rate (eGFR), serum alanine transferase (ALT), serum aspartate transferase (AST), fasting blood sugar (FBS) and platelet (PLT) count were not statistically significant. However, serum urea, serum uric, serum cystatin C (Cys−C), serum total cholesterol (TC), serum triglycerides (TG), whiteblood cell count (WBC) and urine protein were significantly higher in patients while total serum protein (TP), serum albumin (ALB), serum prealbumin (PALB), serum albumin-globulin ratio (A/G), red blood cell count (RBC) and hemoglobin concentration (HGB) were significantly reduced (Table 1).

3.2. Comparison of urine markers of tubular injury between patients and healthy controls, and between patients with and without nephrotic syndrome

The urine concentrations of NAG, RBP, KIM-1 and NGAL in patients and healthy controls, and in patients with and without nephrotic syndrome were compared as shown in Table 2. All biomarkers were significantly increased in the patients while NAG, KIM-1 and NGAL but not RBP were significantly increased in patients with nephrotic syndrome.

Table 2
Comparison of urine markers of tubular injury between patients and healthy controls, and between patient with and without nephrotic syndrome.

Urine markers of tubular injury	Comparisons between patients and health controls			Comparisons between proteinuria grades.		
	IMN patients (165)	Healthy controls (64)	P-value	Subnephrotic range (86)	Nephrotic range (79)	P-value
NAG/Ucr (IU/mmol)	1.53 (0.86–2.75)	0.38 (0.30–0.52)	< 0.001	0.98 (0.59–1.56)	2.36 (1.60–5.00)	< 0.001
RBP/Ucr (mg/mmol)	0.90 (0.42–1.52)	0.02 (0.01–0.02)	< 0.001	1.01 (0.56–1.85)	0.710 (0.35–1.40)	NS
KIM-1/Ucr (μg/mmol)	177.67 (87.65–396.94)	39.48 (26.35–55.52)	< 0.001	117.76 (64.90–306.19)	260.51 (130.31–518.20)	< 0.001
NGAL/Ucr (mg/mmol)	3.41 (1.74–8.24)	1.58 (0.77–2.18)	< 0.001	2.73 (1.37–4.63)	5.64 (2.56–13.35)	< 0.001

NAG: *N*-acetyl-β-D-glucosaminidase, RBP: retinol-binding protein, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatianse-associated lipocalin, UCr: urine creatinine, IMN: idiopathic membranous nephropathy.

Table 3
Correlation of urine markers of tubular injury and selected parameters of renal function in idiopathic membranous nephropathy.

Renal parameters	NAG		KIM-1		NGAL		RBP	
	r	p-value	r	p-value	r	p-value	r	p-value
UP	0.678	< 0.001	0.313	< 0.001	0.404	< 0.001	−0.107	NS
Serum Cr	0.345	< 0.001	0.193	0.015	0.117	NS	−0.092	NS
Serum urea	0.222	0.005	0.230	0.004	0.12	NS	−0.047	NS
Serum uric acid	−0.184	0.022	−0.018	NS	−0.259	0.001	−0.066	NS
Serum Cys-C	0.345	< 0.001	0.362	< 0.001	0.281	< 0.001	−0.095	NS
eGFR	−0.335	< 0.001	−0.229	0.004	−0.335	< 0.001	0.001	NS

NAG: *N*-acetyl-β-D-glucosaminidase, RBP: retinol-binding protein, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatianse-associated lipocalin, eGFR: estimated glomerular filtration, Cys−C: cystatin C, Cr:creatinine, IMN: idiopathic membranous nephropathy, r: correlation coefficient, UP: urine protein.

3.3. Assessment of correlation between urine markers of renal tubular injury and selected parameters of renal function in idiopathic membranous nephropathy

In order to establish whether the urine markers of renal tubular injury are linearly related with the severity of IMN, we determined the correlation of each biomarker with major parameters renal function including proteinuria, eGFR, serum creatinine, urea, uric acid and Cys−C. As shown in Table 3, urinary NAG was significantly correlated with all renal parameters. Urinary KIM-1 correlated significantly with all parameters except serum uric acid while urinary NGAL correlate significantly with proteinuria, uric acid, Cys-C and eGFR but not serum creatinine and urea. No significant correlation was observed between urinary RBP and any of the renal parameters.

3.4. Assessment of urine concentrations of tubular injury markers in patients with different histological grades of tubular injury

To assess whether a relationship exists between histological grades of renal tubular injury and the concentration of urine markers, we grouped the patients into two groups according to histogical grades and compared the biomarker concentrations. Being the majority ($N = 99$), patients with mild histological were considered as one group while the other patients with moderate and severe histological grades ($N = 61$ and $N = 5$ respectively) were combined to form another group.

As shown in Table 4, all urine markers (except RBP) tended to increase in the group with moderate and severe grades of histological tubular injury. However, no biomarker was significantly increased among patients with moderate and severe histological grades of tubular injury.

3.5. Comparison of renal function at different histological grades of renal tubular injury

To establish the presence of significant differences in renal function between patients with mild histological grade and those with moderate and severe grades, we compared renal parameters i.e., urine protein, serum creatinine, Cys−C, total protein, urea, uric acid and eGFR between the 2 groups as shown in Table 5. Although the general trend suggested lower renal function in the group with moderate and severe histological grades, no significant differences were observed in the majority of renal parameters. Only serum creatinine and Cys-C were significantly increased in the group with with histological grades 1 and

Table 4
Comparison of urine markers of renal tubular damage in different histological grades.

Urine markers of renal tubular injury	Histological grades of tubular injury		P-value
	Mild (99)	Moderate and severe (66)	
NAG/Cr (IU/mmol)	1.31 (0.80–2.46)	1.78 (0.94–3.23)	0.220
URBP/Cr (mg/mmol)	0.92 (0.45–1.50)	0.84 (0.38–1.53)	0.735
KIM-1/Cr (μg/mmol)	161.38 (85.85–324.90)	219.88 (91.37–502.62)	0.387
NGAL/Cr (mg/mmol)	3.04 (1.64–7.11)	3.675 (2.19–8.91)	0.304

NAG: *N*-acetyl-β-D-glucosaminidase, RBP: retinol-binding protein, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatianse-associated lipocalin, Cr: creatinine.

Table 5
Comparison of renal parameters between patient groups of different histological grades of tubular injury.

Clinical parameters of renal function	Histological grades of tubular injury		p-value
	Mild (99)	Moderate and severe (66)	
Urine protein (g/l)	2.79 (1.19–5.04)	3.40 (1.96–6.70)	NS
Serum creatinine (μmol/l)	65.65 (53.57–74.72)	69.5 (57.40–80.80)	0.037
Serum Cys-C (mg/l)	1.17 (0.96–1.36)	1.24 (1.10–1.47)	0.039
eGFR (ml/min*1.73 m^2)	104.91 (97.44–113.38)	102.00 (89.20–113.38)	NS
Serum total protein (g/l)	47.11 ± 8.66	45.08 ± 9.56	NS
Serum albumin (g/l)	26.63 ± 7.29	25.06 ± 7.52	NS
Serum urea (mmol/l)	5.99 ± 2.03	6.44 ± 2.04	NS
Serum uric acid (μmol/l)	327.22 ± 88.09	337.80 ± 99.78	NS

eGFR: estimated glomerular filtration rate, Cys-C: Cystatin C.

Table 6
Urine concentrations of tubular injury markers in patients with distinct renal functions.

Urine markers of renal tubular injury	eGFR		P-value
	$< 90\ ml^{-1}.min^{-1}.1.73\ m^{-2}$ (34)	$> 90\ ml^{-1}.min^{-1}.1.73\ m^{-2}$ (131)	
NAG/Cr (IU/mmol)	3.44 (1.47–5.34)	1.31 (0.72–2.12)	< 0.001
RBP/Cr (mg/mmol)	0.80 (0.35–1.99)	0.90 (0.42–1.47)	NS
KIM-1/Cr (μg/mmol)	410.56 (133.15–875.75)	144.65 (81.84–310.10)	< 0.001
NGAL/Cr (mg/mmol)	12.71 (3.00–26.45)	3.02 (1.58–5.24)	< 0.001

NAG: *N*-acetyl-β-D-glucosaminidase, RBP: retinol-binding protein, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatianse-associated lipocalin, Cr: creatinine, eGFR: estimated glomerular filtration rate.

2 of renal tubular injury.

3.6. Comparison of tubular injury markers between patients with distinct renal functions

To ascertain whether the markers of tubular injury are sensitive to renal function, we grouped patients into two groups with distinct renal functions according to the eGFR. In this study, patients with eGFR < 90 ml/min*1.73 m^{-} exhibited a significant difference from those whose eGFR was > 90 ml/min*1.73m^2 i.e., 70.03 (54.90–81.50) ml/min*1.73m^2 versus 106.4 (101.50–114.36) ml/min*1.73m^2, p < .001. So, we compared the urine concentrations of tubular injury markers between the 2 groups as shown in Table 6. With exception of RBP, all urine other markers were significantly increased in patients with eGFR. Lower than 90 ml/min*1.73m^2.

4. Discussion

In spite of being primarily a glomerular disease, IMN's pathology reveals both glomerular and renal tubular abnormalities on a renal biopsy. This notwithstanding, the role of urine markers of tubular injury in the management of IMN have remained poorly appreciated. For long, research has dwelt on the importance tubular injury in renal diseases other than IMN. The role of urine markers of tubular injury have been considerably studied in diabetic nephropathy and Immunoglobulin A nephropathy [12–16]. Little or no attention has been paid to IMN in spite of being a major cause of adult nephrotic syndrome and having an increasing prevalence [17]. To assess their potential in IMN, we determined and compared the concentrations of urinary NAG, NGAL, KIM-1 and RBP between patients and healthy subjects and assessed their capabilities to reflect the histological grades of tubular injury in IMN. We also determined their relationship with parameters of renal function such as eGFR, serum creatinine, uric acid urea, Cys-C and proteinuria.

Urinary RBP was significantly higher in patients compared to healthy controls (p < .001). However, its concentration among patients did not correlate significantly with any parameter of renal function (Table 3) including proteinuria, on which the prognosis and severity of IMN are premised. The observed increase of urinary RBP in IMN over healthy controls is an indicator of its capability to reflect renal tubular injury as previously reported in chronic kidney disease (CKD) [18]. However, its insignificant difference between patients with and without nephrotic syndrome coupled with the lack of significant correlation with all parameters of renal function suggests poor or total lack of a prognostic potential for IMN, despite the previous studies that suggested so in other kidney diseases [14,19].

RBP is a low molecular weight protein freely filtered through the glomerular membrane. Its urinary concentration is kept low by tubular re-absorption which occurs unimpeded under physiological conditions. However, in renal diseases characterized by tubular injury (as is the case with IMN), its re-absorption is impaired causing a rise in the urinary concentration.

The concentration of urinary NAG was significantly increased in patients compared to the health controls (p < .001) and in patients with nephrotic syndrome compared to those without nephrotic syndrome (p < .001). Furthermore, NAG was significantly correlated with not only eGFR, serum creatinine, uric acid, urea and Cys−C, the general indicators of renal dysfunction but also with proteinuria, a prognostic indicator of IMN (Table 3). These results of this study suggested urinary NAG as capable of reflecting renal tubular injury and the rate of renal function decline. Our results are consistent with the Bazzi et al. study in which high concentration of urine NAG was found capable of identifying IMN patients with poor prognosis at the time of diagnosis [20].

NAG is a high molecular weight lysosomal enzyme abundantly expressed in the proximal tubular epithelium. It is secreted in small amounts under normal conditions maintaining a low urinary concentration. However when the renal tubular epithelium is damaged, its secretion is enhanced causing significant urinary increase both in content and activity.

Urinary KIM-1 was significantly increased in patients compared to the healthy controls (p < .001) and in patients with nephrotic syndrome compared to those with nephrotic range proteinuria (p < .001). It was also significantly correlated with eGFR, serum creatinine, urea and Cys-C (Table 3), the general indicators of renal dysfunction and proteinuria, a parameter for IMN prognosis. It also exhibited a positive association with proteinuria and a negative correlation with eGFR. Presently, however, its roles in the management of IMN are not yet elucidated as very few studies have been undertaken to investigate its roles in IMN. Our results are consistent with the Maas et al. study [21], and the non IMN studies in which KIM-1 was found with a prognostic potential [22,23].

KIM-1 is a trans-membrane protein abundantly expressed only in damaged proximal tubular epithelial cells. A high concentration of urinary KIM-1 is indicative of renal tubular damage especially in the early stages of renal diseases.

Like NAG and KIM-1, urinary NGAL was increased in patients compared to the healthy controls (p < .001) and its concentration in patients with nephrotic range proteinuria was significantly higher in relation to those with subnephrotic range proteinuria (p < .001). Urinary NGAL was also significantly correlated with the general parameters of renal function except serum urea and creatinine (Table 3). Accordingly, these results suggest NGAL as capable of reflecting the severity of renal tubular injury in IMN. Our findings are consistent with the results of Bolignano et al. [24] in which urinary NGAL was significantly increased and correlated with proteinuria in various glomerular diseases. Being an acute phase protein, the clinical relevance of NGAL has mainly been studied in acute kidney diseases [15,25–27]. Recently however, its significance in CKD especially IgA and diabetic nephropathies [21,28–31] has been appreciated but its potential for IMN has not yet been elucidated.

Taken together, the findings of this study confirm that the urine markers of renal tubular injury are significantly increased in IMN patients. All patients included in this study exhibited renal tubular injury ranging from mild to severe histological grades as per biopsy results. However, no significant differences in the urine biomarker concentrations were observed (Table 4) probably due to lack of significant

differences in renal function of the histological groups under comparison (Table 5). Only serum creatine and Cys-C were significantly increased in patients with moderate and severe histological grades of tubular injury while other parameters were marginally increased.

Although these results suggest that urine markers do not reflect histological grades of tubular injury, a comparison between patients with distinct renal functions revealed significant differences in all except RBP (Table 6). It is thus plausible to speculate that NAG, NGAL and KIM-1 could still hold a potential of discriminating histological grades especially in patients with distinct renal functions.

The limitations of this study include a small number of patients with a severe histological tubular injury (only 5) and the absence of a clear-cut distinction in renal functions between patients with mild and moderate histological grades. Furthermore, the lack of disease controls did not permit the investigation of biomarkers' potential in other primary glomerular diseases while the failure to screen patients for IMN's pathogenic antibodies did not permit assessment of antibodies' effect on urine biomarker concentrations. Accordingly, future studies should further investigate the potential of urinary NAG, NGAL and KIM-1 in patients with distinct renal functions taking all these variables into account.

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