sciendo
10.2478/AMB-2018-0013

COMPARATIVE ASSESSMENT OF uNGAL, uNAG AND CYSTATIN C AS EARLY BIOMARKERS IN RENAL POST-TRANSPLANT PATIENTS

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Abstract. *Urinary neutrophil gelatinase-associated lipocalin (uNGAL), urinary N-acetyl-b-d-glucosaminidase (NAG), urinary α1-microglobulin/creatinine ratio and cystatin C have been suggested as potential early markers of delayed graft function (DGF) following kidney transplantation. We conducted a prospective study in 50 consecutive kidney transplant recipients to evaluate serial changes of these biomarkers within the first week after transplantation and assess their performance in predicting DGF (dialysis requirement during initial post-transplant week) and graft function throughout the first year. Urine samples were collected on post-transplantation days 0, 1, 2, 4, and 7. Statistical analysis: Linear mixed and multivariable regression models, receiver-operating characteristic (ROC), and areas under ROC curves were used. At all-time points, mean urinary NGAL levels were significantly higher in patients developing DGF. Shortly after transplantation (3-6 h), uNGAL and uNAG values were higher in DGF recipients (on average +242 ng/mL; NAG – 6.8 U/mmol creatinine, considering mean dialysis time of 4.1 years) and rose further in the following days, contrasting with prompt function recipients. On Day-1 uNGAL levels accurately predicted DGF (AUC-ROC = 0.93), with a performance higher than serum creatinine (AUC-ROC = 0.76), and similar to cystatin C (AUC-ROC = 0.95). Multivariable analyses revealed that uNGAL levels at days 4 and 7 were strongly associated with one-year serum creatinine level. Urinary NGAL, serum cystatin C is an early marker of graft injury and is independently associated with dialysis requirement within one week after transplantation and one-year graft function.*

Key words: *urinary NGAL, NAG, cystatin C, kidney transplantation*

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INTRODUCTION

Early determination of allograft function and prognosis could lead to the development of therapies for kidneys with significant ischemia-reperfusion injury (IRI) and more effective recipient management, thereby improving outcomes. Unfortunately, the use of baseline characteristics alone (donor and recipient age, etc.) has limited accuracy in predicting outcomes. Recent insights regarding IRI biomarkers in the setting of acute kidney injury (AKI) suggest allograft injury and recovery could be better characterized by these biomarkers. Delayed graft function (DGF) is an important complication of

kidney transplantation (KT) that adversely affects allograft survival. Despite substantial improvements in the field of KT, the incidence of DGF is rising with the growing practice of accepting expanded criteria donors to increase transplantation rates [1-6]. Delayed graft function predisposes kidney graft to acute and chronic rejection, contributes to progressive allograft dysfunction, and increases the risk of premature graft loss [9-11].

Reliable biomarkers enabling early discrimination of DGF in kidney transplantations are lacking, which impairs timely therapeutic interventions. Traditionally, acute graft dysfunction is diagnosed by measuring the serum creatinine, but this parameter is an unreliable indicator of kidney function during an episode of acute injury [12]. One of the most promising biomarkers of acute kidney injury is neutrophil gelatinase-associated lipocalin (NGAL), which is released into the blood from activated neutrophils during inflammatory processes. In healthy conditions, this lipocalin is found in the urine only in very small amounts. Massive NGAL quantities excreted in the urine (uNGAL) usually indicate damage of proximal tubular cells [14-15]. Graft injury due to ischemia-reperfusion is an inevitable consequence of KT procedure and can result in varying degrees of early graft dysfunction, which can be considered a form of post-transplantation acute kidney injury. For this reason, several studies investigated the utility of NGAL for the diagnostic and prognostic of acute graft dysfunction following KT [19, 21, 22]. Recently, the prognostic value of uNGAL on graft function in one year after transplantation was also examined and has presented conflicting findings [23, 25, 26].

In order to support the usefulness of uNGAL, uNAG and serum cystatin C as reliable markers of graft injury and to clarify the role of these promising biomarkers in the prediction of kidney function beyond the first week after transplant, we conducted a prospective study to: (a) evaluate longitudinal changes of uNGAL and uNAG levels over the first week after KTx and identify factors associated with these changes; (b) assess the performance of uNGAL and uNAG in predicting DGF (defined as the requirement for dialysis within the first 7 days after transplantation); (c) appraise the long-term prognostic value of urinary NGAL,NAG and alfa-1-microglobulin/creatinine ratio measured within one week post transplantation on kidney allograft function, evaluated by one-year of serum cystatin C and serum creatinine. AKI due to DGF, defined as the need for dialysis within the first week after transplantation, complicates 4-10% of live donor and 5-50% of cadaveric kidney transplants [28]. DGF predisposes the graft to acute rejection [29], and increases the risk of chronic allograft nephropathy and graft loss and is an independent risk factor for suboptimal graft function in one year after transplantation. A variety of clinical parameters have been proposed for the prediction of DGF based on the preoperative risks, but unfortunately no objective and reliable markers exist for early diagnosis of DGF. Several clinical parameters for the diagnosis of DGF have been applied based on urine output, the rate of creatinine reduction and the need for hemodialysis, but these clinical variables may take several days to be confirmed. Consequently, therapeutic interventions that may ameliorate DGF have been ineffective in human studies [2], at least in part, due to the paucity of early markers for renal dysfunction. Tools for the early diagnosis of DGF following kidney transplantation have been sparse. Consequently, there has been a marked interest in the potential use of early protocol biopsies for the diagnosis, quantitation, and prediction of injury following cadaveric transplantation. For example, the number of apoptotic tubular cells in donor biopsies either before [6] or immediately after engraftment [7] has been shown to correlate with early allograft function. Application of the novel biomarkers of AKI (NGAL, NAG cystatin C) for detection of early allograft dysfunction may prove useful. They could predict the trend in serum creatinine in the post-transplant period without the need for biopsy [8], which allows early initiation of treatment to improve the long-term prognosis.

Human Neutrophil Gelatinase Associated Lipocalin (NGAL) is one of the most extensively studied novel biomarkers both in AKI and in kidney transplant dysfunction. It was originally identified as a component, along with gelatinase (MMP-9), of a disulfide-linked heterodimer secreted by neutrophils [21]. NGAL can also be secreted from neutrophils as both a 25 kDa monomer and as a 46 kDa disulfide-linked dimer in the absence of associated gelatinase [16]. It is normally expressed at very small levels in several human tissues, including kidneys, lungs, stomach and colon. NGAL expression is markedly elevated following renal ischemia/reperfusion injury (I/R). It is freely filtered by the glomerulus, completely absorbed by the proximal tubule. AKI causes failure of absorption of the filtered NGAL, and more importantly, increased synthesis of NGAL by the distal nephron comprising the major fraction of urinary NGAL. The injured kidney is not the major source of plasma NGAL, but surprisingly, the distant organs mainly the liver and the lungs, which showed increased expression of NGAL mRNA. Its expression is induced in the proximal tubules during the regeneration process after kidney injury. Low urinary excretion of NAG is helpful in the di-

agnosis of kidney transplant rejection as the amount of excreted NAG depends on the graft mass, and the amount of urinary creatinine depends on the recipient body mass, a low NAG excretion (related to urinary creatinine) could be a surrogate marker of an unfavorable low graft to body-weight ratio, which in turn might be associated with a reduced graft survival.

Cystatin C is a proteinase inhibitor with a low molecular weight. It is produced at a constant rate in all nucleated cells investigated to date and released into plasma, freely filtered by the glomeruli and nearly completely metabolized in the proximal tubules. The concentration of serum cystatin C is mainly determined by glomerular filtration, which makes cystatin C an ideal endogenous surrogate marker of kidney function and estimation of GFR [30, 31]. It has been shown to be superior to serum creatinine [31]. The production of cystatin C has been extensively reported to be independent of and unaffected by sex, age, height, weight, and muscle mass [24]. Cystatin C levels have been found to be influenced by abnormal thyroid function, use of immunosuppressive therapy and the presence of systemic inflammation [29]. Serum cystatin C concentrations have demonstrated good inverse correlations with radionuclide-derived measurements of GFR. The diagnostic value of cystatin C as an estimate of GFR has now been investigated in multiple clinical studies [29]. There is a suggestion that cystatin C-based estimates of GFR may perform better in selected patient populations, in particular those with lower serum creatinine concentrations such as elderly patients, children, renal transplant recipients, cirrhotics and those that are malnourished.

MATERIAL

The secondary urine samples are taken every day (if necessary twice daily), they are processed chemically, biochemically, and after centrifugation are considered elements of sediment. In the morning 10 ml urine samples were collected for evaluation of low-molecular proteins (alfa-1-microglobulin), NGAL, NAG and creatinine, and stored at 40 °C until analysis. Samples were analyzed daily from day 0 before the surgery 1, 2, 3, 4, 7 and 31 days after surgery. From the day of the surgery, allograft function was assessed daily with special attention in the first 0, 1, 2, 4, 7 days. The first sample of blood after the operation was received immediately when patients arrived at the intensive care unit, i.e. average delay of 1 hour 52 min. ± 6 minutes after the end of the operation. Samples of blood were always taken at 08:00 pm every day for the next 31 days.

METHODS

Urine samples for urinary NGAL, NAG and alfa-1-microglobulin/creatinine ratio determination were collected 3 to 6 h after surgery (0 or baseline); on the following morning, nearly 8 to 12 h after graft reperfusion (first day); and then on the second [2], fourth [4], and seventh day [7], for a total of five samples for each patient. The same laboratory technician, who was blinded to patient information, performed urinary NGAL measurements using a two-step chemiluminescent microparticle immunoassay on a standardized clinical platform (ARCHITECT, Abbott Diagnostics).

Urinary N-acetyl-β-D-glucosaminidase (NAG) activity was measured in urine by colorimetric assay (Boehringer Manchaim, Germany). This method is using the substrate 3-cresolsulfonphaleinyi-N-acetyl-β-D-glucosaminidine-Na salt, which is hydrolysed in the urine sample by the action of NAG. Serum creatinine levels were determined preoperatively, daily, until hospital discharge, after transplantation to evaluate later graft function. Serum creatinine measurements were performed by Jaffé method (Roche Diagnostics). Cystatin C was measured with a particle enhanced immunonephelometric method (Siemens Diagnostics) at the same time points as uNGAL, except for baseline values.

STATISTICAL ANALYSIS

Data is presented as mean ± SD or as median and is ranged when appropriate after checking for Gaussian distribution. Differences between the two groups were evaluated by the Wilcoxon signed-rank test. Multiple comparisons were performed by the Friedman repeated-measure ANOVA on ranks followed by the Dunn test. Correlation between techniques was evaluated by linear regression and ANOVA. Results with $P < 0.05$ were considered statistically significant.

RESULTS

During time recruitment 50 patients were enrolled. Baseline data are showed in Table 1.

At first urine samples were obtained from 48 patients. In the following days, urine samples were collected from 42 patients on the 1-st, 2-nd, 4-th and seventh day. The samples have been examined for serum creatinine and cystatin C and NGAL as NAG in the urine in patients before transplantation and the first following week, was in a stable and delayed graft function (Table 2).The concentration of these biomarkers before transplantation was with particularly high values,

corresponding to a patient placed on chronic hemodialysis program. In the first days after transplantation the patients who had after transplantation investigated biomarkers showed concentrations with tendency toward normalization. Transplant patients with DGF in this period maintained high levels of creatinine and uNGAL, which was the reason to part with them for the application of dialysis (biopsy was performed in five patients).

Receiver-operating characteristic (ROC) curves showed that urine NGAL in the first postoperative days were accurate in predicting for DGF. The table shows the resulting sensitivity, specificity, and predictive values for urine NGAL at concentrations of exclusion, which provided the maximum amount of sensitivity and specificity. In terms of areas under the curves ROC (AUC), the ability of urine NGAL to predict DGF is moderately right at the beginning and the first day and very accurate in the second, fourth and seventh day (Table 4). In the first two days after the transplantation, the diagnostic performance of urine NGAL is better than serum creatinine and quite similar to that of cystatin C. Serum creatinine reduction between the first and second days shown with AUC = 0.58 (0.64-0.92) is a bad representation of urine NGAL for the prediction of TFP.

The changes in the concentration of urinary NGAL are shown in Table 5. High concentration of urinary NGAL was confirmed on the first day of transplantation and it showed tendency to decrease afterwards. Between the two groups of patients were found significant, although not so pronounced, changes in creatinine levels. Moreover, we confirmed that glomerular filtration rate of patients with stable graft function significantly improved after three months of follow-up.

Table 1. Summary of baseline and clinical characteristics in kidney transplant donor and recipients samples

	DGF	SGF
DONOR		
Age(yr)	47.9 ± 12.3	48.6 ± 11.8
Living donor		
Serum creatinine μmol/L	81.0 ± 18.0	78.0 ± 16.0
Donor-recipient		
Cold ischemia, time(h)	13.6 ± 7.9	9.6 ± 7.3
Recipient		
Age (yr)	52.8 ± 11.6	42.8 ± 14.2
BMI (kg/m^2)	26.2 ± 4.4	23.6 ± 5.0
Time of dialysis(yr)	6.4 ± 3.8	4.9 ± 2.3
Cause of kidney disease	3	8
IgG nephropathy	3	8
Glomerulonephritis	6	2
Diabetic Nephropathy	15	13
Autosomal dominant polycystic kidney	14	10
Unknown	5	3

Table 2. Levels of NGAL and serum creatinine in urine during the first week after transplantation, according to graft function (DGF or SGF)

Urine NGAL ng/ml	3-6 h after transplantation	1 day	2 day	4 day	7 day
DGF	65 7(328-1648)	895 (505-1156)	834 (502-2232)	845 (512-1569)	421 (106-1159)
SGF	236 (101-423)	132 (60-285)	85 (35-142)	42 (32-89)	31 (25-51)
Serum creatinine μmol/L	Before transplantation				
DGF	663.0 (530-1032)	724.0 (486-822)	663.0 (521-751)	609.9 (139-707)	565.7 (468-786)
SGF	689.5 (450.2-830.9)	556.9 (406-698)	380.1 (247-539)	221.0 (141-282)	167.6 (123-212)

Table 3. Levels of NGAL and serum cystatin C in urine during the first week after transplantation, according to graft function (DGF or SGF)

Urine NAG U/mmol creatinine	3-6 h after transplantation	1 day	2 day	4 day	7 day
DGF	8.6 ± 2.9	9.2 ± 1.8	13.5 ± 3.2	14.9 ± 5.1	10.3 ± 2.2
SGF	5.8 ± 2.3	6.2 ± 3.1	5.4 ± 1.8	4.3 ± 1.5	4.1 ± 1.4
Serum cystatin C mg/L	3-6 h after AR				
DGF	5.3 ± 1.4	7.3 ± 2.6	8.4 ± 1.6	7.3 ± 1.9	6.8 ± 1.5
SGF	3.8 ± 1.2	4.6 ± 1.9	4.5 ± 1.5	3.7 ± 1.3	3.2 ± 1.4

Table 4. Area under the receiver-operating characteristic curve at each time point for urinary NGAL and serum cystatin C for predicting DGF

	Time after transplantant	AUC (95% CI)	p
	h after OP	0.70 (0.53-0.91)	
	0h	0.68 (0.55-0.81)	
	6h	0.81 (0.70-0.92)	0.010
	12h	0.76 (0.64-0.88)	
	18h	0.78 (0.67-0.89)	
	1 day	0.80 (0.73-0.98)	< 0.001
NGAL ng/ml	2 day	0.88 (0.84-0.95)	< 0.001
	4 day	0.91 (0.65-0.98)	< 0.001
	7 day	0.90 (0.85-1.0)	< 0.001
Serum cystatin C mg/L	2 day	0.92 (0.81-0.97)	< 0.001
	4 day	0.93 (0.86-1.0)	< 0.001
	7 day	0.92 (0.80-1.0)	< 0.001

Table 5. uNGAL, serum creatinine and expected results for GFR at a level of an allograft function after transplant

	Time after transplantation	DGF	SGF	P
Urinary NGAL (ng/l)	0 h	463	254	0.015
	6 h	612	322	< 0.001
	12 h	852	301	< 0.001
	18 h	1023	215	< 0.001
	First day	1010	235	< 0.001
	Second day	755	113	< 0.001
Serum creatinine μmol/l – after 3 months		198	156	< 0.01
GFR (ml.min.1.73 m^2) – after 3 months		45.8 (24-80)	52.9 (39-82)	< 0.001

DISCUSSION

Similar to other areas in medicine, in kidney transplantation early diagnosis and timely intervention could improve outcomes. If DGF could be detected in the early hours after surgical procedure, maybe a tailored and more individualized intervention could be achieved. The major finding of this study is that uNGAL is a promising biomarker for allograft dysfunction that can be easily and noninvasively assayed in the early post-transplant period. We prospectively evaluated uNGAL of 40 kidney allograft recipients during the first post-transplant week. At all measured time points, uNGAL levels were consistently higher in

patients who developed DGF, including the earliest levels obtained from the first urine sample collected approximately 3 to 6 h after transplant surgery. At this time, clinical diagnosis of DGF is yet not possible, but a simple and noninvasive test can already recognize kidney dysfunction and stratify patients according to likelihood of requiring post-transplant dialysis.

It would be ideal to diagnose graft dysfunction with an early and highly sensitive biologic marker of renal tubular injury. One of the most promising markers is NGAL, and our findings provide further information for the use of uNGAL as a diagnostic and prognostic tool for DGF. According to our estimation, uNGAL values shortly after transplant surgery will be much higher in patients who went on to develop DGF and will rise further in the following days. In contrast, patients with prompt function will have lower levels, which will decrease consistently during the week. The dynamics of changes in these recipients compared to those who presented DGF is quite different. It seems that, not only the baseline levels, but also the pattern of uNGAL longitudinal changes can reflect graft dysfunction. Hollmen et al. found initial levels of uNGAL higher in DGF patients, but on the following day a decrease was observed, as it happened with recipients with prompt function [23]. As mentioned before, our study did not confirm this declining in DGF patients. Recipients who went on to develop DGF had initial higher levels of uNGAL that rise further in the following post transplant days, differing from patients with prompt graft function. Our findings are in agreement with results reported by Hall et al. [24]. It seems that, above and beyond the markedly higher levels of uNGAL in patients with graft dysfunction, the contrasting pattern of uNGAL longitudinal changes can distinguish recipients who will need dialysis in the first week after transplantation. In accordance with previously published data [19, 20, 22], we confirmed the good performance of NGAL in predicting graft dysfunction in the early post transplant period. Using ROC analysis, our study also confirms uNGAL as a good diagnostic marker for identifying patients with graft dysfunction and who subsequently will require dialysis. The AUC-ROC for uNGAL was moderately accurate for DGF prediction within the first day after transplantation, and it was excellent on day 2 and day 4.

We report a superior performance of uNGAL levels for predicting DGF over serum creatinine measured at the same time. Urinary NGAL measured on the first day predicted DGF with an AUC-ROC of 0.93, which is markedly better than an AUC-ROC = 0.76 shown by serum creatinine measured on the same day, and also than an AUC-ROC = 0.83 obtained from creatinine reduction ratio from the first to the second day, but quite similar to cystatin C (0.95), a marker considered more accurately to detect changes in renal function [33]. Furthermore, our analyses also revealed that uNGAL levels predicted DGF, even after adjusting for pre-transplant variables known to be traditionally associated with DGF. Besides DGF, the other factors that significantly influenced uNGAL levels were previous time of dialysis, recipient's age at time of transplantation and cold ischemia time. These three variables were positively correlated with uNGAL values. Mishra and coworkers have shown that the immunohistochemical staining intensity of NGAL was strongly correlated with cold ischemia time and NGAL expression was significantly increased in deceased donor biopsies [16]. We found that uNGAL levels were higher in graft recipients from deceased donors, but only significantly higher on the second day.

Similarly to other authors [16, 22], we have chosen to measure NGAL in urine, instead of blood, since uNGAL represents tubule damage in the kidneys rather than filtration from blood. An increased level of NGAL in urine usually indicates injury of proximal tubular cells and seems to be more specific compared to serum NGAL, which can be produced by other organs and released into the circulation following a transplant surgery [12]. However, despite the undoubted value of urinary markers of kidney injury, their use in transplant recipients can be also a drawback because of possible transient graft anuria, which may preclude the availability of urine and consequently the lack of samples needed to measure NGAL.

Since allograft rejection during the first week and year after kidney transplantation leads to persistent allograft dysfunction and reduced long-term graft survival, it is important to define a good early predictor of kidney damage which is less invasive than allograft biopsy. Previously, published data determined the urinary NAG excretion in renal-transplant recipients at different times after transplantation and showed a direct correlation between the extent of urinary NAG excretion and both the time after renal transplantation and prednisolone dosage [45]. Increased levels of urinary NAG in some recipients of kidney transplantation are due to the administration of calcineurine inhibitors [44]. In our study, we evaluated NAG activity in urine samples collected during the first year after transplantation. We found no impact of NAG activity in day 1 urine samples on the long-term allograft function. However, levels of this enzyme were higher in patients with DGF. Lack of immediate function of the transplanted kidney usually results from ATN, which mainly affects the proximal tubules. These cells are characterized by high metabolism, and

therefore, are especially jeopardized by the ischemia (Burne-Taney et al., 2005) [37]. It seems that the assessment of proximal tubule damage markers could be an efficient DGF prognostic tool. Matteucci and coworkers (1998) confirmed the correlation of urinary NAG concentration with early allograft function. Kuzniar et al. observed the early post-transplantation period, and recorded higher urinary NAG concentration in patients that developed ATN [44]. High activity of NAG correlated with deteriorating kidney function throughout the whole observation period contributes for better diagnose of DGF. In the first weeks of the post-transplantation period, kidney tubules are exposed to the IRI, toxicity of the immunosuppressive drugs and acute rejection episodes. Damage to the tubules made by those factors determines the long-term function of the allograft (Nankivell et al., 2004). It seems that the most important factor affecting the kidney tubules is IRI. The results of our study confirm that urinary NAG activity in samples collected on the 1st day after transplantation is a predictor of DGF occurrence. High activity of NAG is associated with poor long-term allograft function, grade of proteinuria in the early postoperative period and glomerular atrophy in allograft biopsy samples.

Monitoring of NAG urine activity is useful in evaluation of early proximal tubule damage and predicting the long-term function of transplanted kidneys. Similarly, in our study, the evaluation of NAG at timepoints other than during the early postoperative period was not useful to predict the long-term function of the transplanted kidney. This may be due to the fact that the inflammation process caused by IRI stabilizes in the later period after transplantation, and the high concentration of NAG decreases. The results of our study confirm that urinary NAG activity in samples collected on the 1st day after transplantation is a predictor of DGF occurrence. High activity of NAG is associated with poor long-term allograft function, grade of proteinuria in the early postoperative period and glomerular atrophy in allograft biopsy samples. Monitoring of NAG urine activity is useful in evaluation of early proximal tubule damage and predicting the long-term function of transplanted kidneys.

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