

## N-acetyl-beta-glucosaminidase urine activity as a marker of early proximal tubule damage and a predictor of the long-term function of the transplanted kidneys

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**Introduction:** Ischaemia-reperfusion injury (IRI) is a factor leading to the damages of the transplanted kidney, what affects mainly the proximal tubules. Early monitoring of tubule damage can be an efficient tool to predict the allograft dysfunction. Present in proximal tubules, N-acetyl-beta-glucosaminidase (NAG) is a lysosomal enzyme whose excretion rises as a result of IRI or acute rejection. The aim of this study was to monitor the NAG urine activity to evaluate the early proximal tubule damage, and to try to predict the long-term function of the transplanted kidney. **Material and methods:** The study enrolled 87 Caucasian renal transplant recipients (61.7% males, 38.3% females, mean age 45.56±14.34 years). Urine samples were collected for NAG and creatinine analysis on the 1st day after transplantation, and then in the 3rd and 12th month. Protocol biopsies were performed in the 3rd and 12th month. **Results:** A significant positive correlation between NAG urine activity in the 3rd month after transplantation and creatinine concentration on the 14th ( $p=0.004$ ) and 30th day ( $p=0.05$ ), in the 3rd month ( $p=0.009$ ) and after the 1st ( $p=0.005$ ) and 2nd year ( $p=0.003$ ) was observed. A statistically significantly higher urinary NAG activity in samples collected in the first 3 days and in the 3rd month after transplantation among patients with DGF ( $p=0.006$  and  $p=0.03$  respectively) was found. There was a significant positive correlation between NAG urine activity in the 3rd month and the grade of tubular atrophy in specimens collected in the 3rd ( $p=0.03$ ) and 12th ( $p=0.04$ ) month. **Conclusions:** Monitoring of NAG urine activity is useful in the evaluation of early proximal tubule damage and predicting the long-term function of the transplanted kidneys.

**Key words:** allograft, CAD, DGF, kidney function, NAG.

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### INTRODUCTION

According to the United Network for Organ Sharing (UNOS) records, 40% of renal allografts are lost during the first decade after transplantation (Cecka, 2002). This is associated with two factors: death with a functioning kidney and chronic allograft dysfunction. Glomerulosclerosis, tubular atrophy and interstitial fibrosis (TA/IF)

damage the transplanted organ, causing kidney function deterioration. Development of allograft histopathological changes involves both immunological and non-immunological factors (Kłoda *et al.*, 2010; Domański *et al.*, 2013). Early detection of TA/IF allows clinicians to slow down or even stop the chronic dysfunction of the transplanted kidney. Ischaemia-reperfusion injury (IRI) is the earliest occurring factor damaging the organ, affecting mainly the proximal tubules. It is considered that IRI is responsible not only for acute lesions, but also for triggering a chronic inflammation process leading to TA/IF (Domański *et al.*, 2013). It was observed that the exposure of the proximal tubules to ischaemia causes transmigration of mononuclear cells and promotes inflammatory infiltration. This process initiates subsequent fibrosis as a result of profibrotic cytokines secretion and stimulation of epithelial-mesenchymal transformation (Burne-Taney *et al.*, 2005; Liu, 2004). Because TA/IF begins in the proximal tubules, early monitoring of tubule damage could be an efficient tool to predict allograft dysfunction.

N-acetyl-beta-glucosaminidase (NAG) is a lysosomal enzyme present in the proximal tubules of the kidney, normally secreted in small concentrations as a consequence of a natural exocytosis process (Price 1992). It was shown that after administration of cadmium, contrast agents, aminoglycosides and other nephrotoxic drugs, excretion of NAG increases (Sanchez-Bernal *et al.*, 1991; Price, 1992; Pergande *et al.*, 1994). Concentrations of this enzyme are also higher in glomerulopathy (Hultberg & Ravnskov, 1981; Perez-Blanco *et al.*, 1997). The levels of NAG were analysed after kidney transplantation as a marker of IRI and acute rejection of the allograft (Kuźniar *et al.*, 2006; Peto *et al.*, 2005). However, NAG was not considered as a marker of long-term kidney allograft function. Therefore, the aim of this study was to monitor NAG urine activity to evaluate early proximal tubule damage and to try to predict the long-term function of the transplanted kidney.

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**Abbreviations:** CAN, chronic allograft nephropathy; CIT, cold ischaemia; GFR, glomerular filtration rate; HLA, human leukocyte antigen; IRI, Ischaemia-reperfusion injury; NAG, N-acetyl-beta-glucosaminidase; PRA, panel-reactive antibody; TA/IF, tubular atrophy and interstitial fibrosis

**Table 1. Clinical characteristics of the studied renal transplant recipients.**

Characteristic	N	Median	Range	mean $\pm$ S.D.
Time of observation (months)	87	36	0.5–60	31.8 $\pm$ 21.54
Age (years)	87	49	18–80	18–80
Dialysis before Tx (months)	70	20	16–75	23.8 $\pm$ 18.16
Residual diuresis (ml)	75	300	300–3000	664.84 $\pm$ 857
Weight (kg)	71	72	72–98.5	70.9 $\pm$ 13.01
CIT (hours)	72	20.5	0–42	21.45 $\pm$ 9.01
Mismatch A	70	1	0–2	1.16 $\pm$ 0.7
Mismatch B	70	1	0–2	1.34 $\pm$ 0.68
Mismatch DR	70	1	0–2	0.71 $\pm$ 0.68
HLA points	66	12	2–19	12.34 $\pm$ 4.2
PRA (%)	61	0	0–40	3.44 $\pm$ 7.1

S.D. — standard deviation; Tx — transplantation; CIT — cold ischemia time; PRA — panel reactive antibody

## MATERIAL AND METHODS

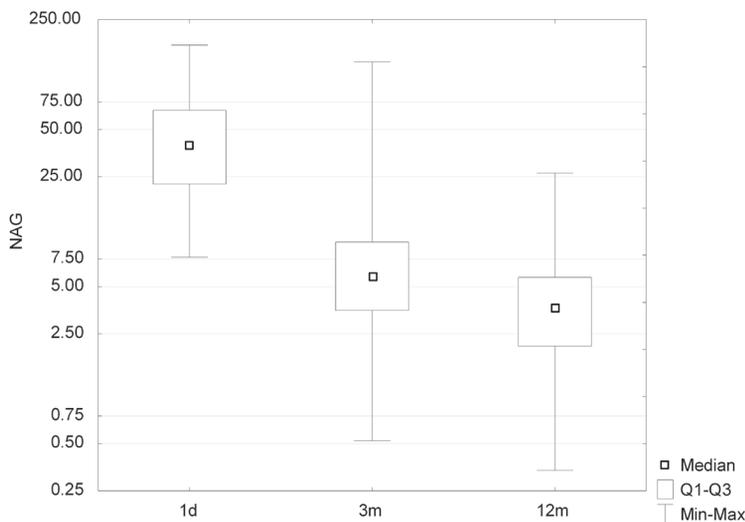
The study enrolled 87 Caucasian renal transplant recipients (61.7% males, 38.3% females, mean age 45.56 $\pm$ 14.34 years, transplantation performed between 2006 and 2008). All patients after kidney transplantation performed between 2006 and 2008 who gave their consent were enrolled in the study. Patients were treated in the Clinical Department of Nephrology, Transplantation, and Internal Medicine of the Pomeranian Medical University in Szczecin, Poland. The average period of observation was 30 months (from 0.5 to 60 months). All patients received a triple immunosuppression regimen: calcineurin inhibitors, mycophenolate mophetil and glucocorticosteroid. Characteristics of studied patients are presented in Table 1. The causes of renal failure were: chronic glomerulonephritis (26.17%), autosomal dominant polycystic kidney disease (16.82%), diabetes (13.08%), inflammatory diseases (9.35%), hypertension (7.48%) and other (9.35%). The cause of renal failure remained unknown in 17.75% of studied

patients. The majority of patients (78%) needed haemodialysis before the transplantation, and the rest of the studied subjects were undergoing peritoneal dialysis (22%).

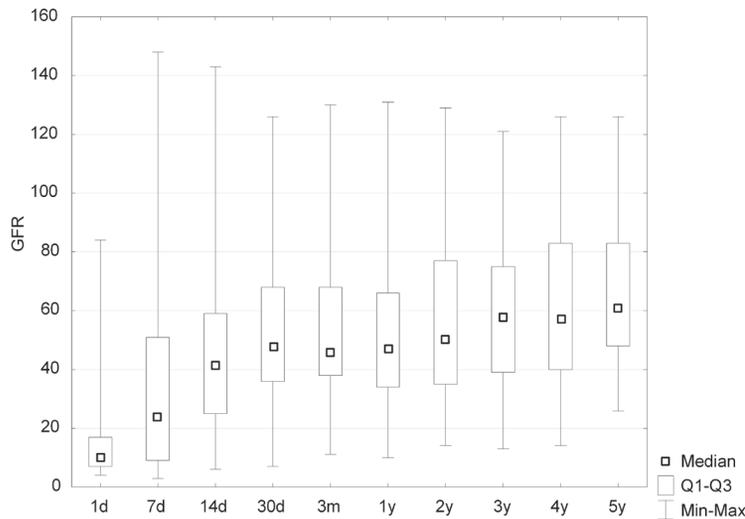
Urine samples were collected for NAG and creatinine analysis on the 1st day after transplantation, and then in the 3rd and 12th month. Among subjects that did not produce urine because of delayed graft function (DGF), the first sample was collected when the amount of urine reached 500 ml per day, which was not later than on the 3rd day after the transplantation. These samples corresponded with the urine samples collected on the 1st day after transplantation from patients without DGF, and they were analysed together. The collected samples were centrifuged at 4000 rpm for 10 min, and urine without the sediment was stored at  $-80^{\circ}\text{C}$  until the time of analysis. The activity of NAG was measured by the Maruhn colorimetric method using *p*-nitrophenyl-N-acetyl- $\beta$ -D-glucosamine (Sigma-Aldrich) as a substrate (Maruhn 1976). The *p*-nitrophenol, which was released in the reaction, was evaluated by measuring the sample compared to the blank sample absorbance at a wavelength of  $\lambda=405$  nm. The concentration of creatinine in the urine was assessed in a reaction with picric acid, after dilution of the urine by 50 times. Activities of NAG and creatinine concentrations in urine were evaluated, and NAG was calculated in relation to creatinine concentration. Moreover, standard urinalysis was performed, and blood samples were collected from all patients to assess creatinine, glomerular filtration rate (GFR), urea, blood count and the level of calcineurin inhibitors at the timepoints of urine sample collection and during the standard follow-up visits. The GFR was calculated by the CKD-EPI Formula using the calculator of the National Kidney Foundation. In addition, the donor data were analysed: the time of cold ischaemia (CIT), human leukocyte antigen (HLA) mismatches, panel-reactive antibody (PRA) and DGF

occurrence. DGF was defined as the need for dialysis within the first week after transplantation. Moreover, part of the observed patients underwent protocol biopsies in the 3rd and 12th month after transplantation. Protocol biopsies were performed to detect clinically silent pathologies of the allograft at an early stage (rejection, immunosuppressive agents nephrotoxicity, polyoma virus infection). Therefore, all patients were offered the protocol biopsies, but they were performed only in patients who gave their consent. Among individuals with delayed graft function, kidney biopsy was performed during the first 2 weeks after transplantation.

**Statistical analysis.** We used Statistica 9 software (StatSoft, Poland) for analysis. As Shapiro-Wilk's test showed that the distributions of NAG activity were significantly different from normal ( $p<0.05$ ), we used non-parametric Mann-Whitney's U test and Spearman's rank



**Figure 1.** The activity of urine NAG during study on 1st day (1d), 3rd month (3m) and 12th month (12m) after transplantation.



**Figure 2.** Patient's glomerular filtration rate (GFR) during study on day 1st (1d), 7th (7d), 14th (14d), 30th (30d), 3rd month (3m) and 1st, 2nd, 3rd, 4th, 5th years (1y, 2y, 3y, 4y, 5y) after transplantation.

correlation test for the statistical analysis. A  $P$ -value  $< 0.05$  was considered as statistically significant.

## RESULTS

The GFR and NAG activity during the study are presented in Figs. 1 and 2. A significant positive correlation between NAG urine activity in the 3rd month after transplantation and serum creatinine concentration on the 14th ( $p=0.004$ ) and 30th days ( $p=0.05$ ), in the 3rd month ( $p=0.009$ ), and after the 1st ( $p=0.005$ ) and 2nd years ( $p=0.003$ ) was observed. Concomitantly, a significant negative correlation between NAG urine activity in the 3rd month after transplantation and GFR on the 14th day ( $p=0.006$ ), in the 3rd month ( $p=0.03$ ), and after the 1st ( $p=0.01$ ), 2nd ( $p=0.01$ ), 3rd ( $p=0.05$ ) and 5th years ( $p=0.07$ ) was observed (Table 2). A statistically significantly higher urinary NAG activity in samples collected in the first 3 days and in the 3rd month after transplantation among patients with DGF ( $p=0.006$  and  $p=0.03$ , respectively) was found.

**Table 2.** Correlation between NAG activity in the 3rd month after transplantation and creatinine and GFR values

NAG in the 3rd month	N	Spearman's R	$p$
GFR 14th day	51	-0.375962	0.006
Creatinine 30th Day	49	0.2758586	0.05
Creatinine 3rd month	51	0.3602091	0.009
GFR 3rd month	51	-0.3080753	0.03
Creatinine 1st year	53	0.3836693	0.005
GFR 1st year	53	-0.3491411	0.01
Creatinine 2nd year	44	0.4313352	0.003
GFR 2nd year	44	-0.3724428	0.01
GFR 3rd year	36	-0.3229254	0.05
GFR 5th year	15	-0.4843612	0.07

$p$  value calculated with Spearman's Rank Order Correlation Test

In regard to histopathological changes in renal allograft biopsies, there was a significant positive correlation between NAG urine activity in the 3rd month after transplantation and the grade of tubular atrophy in specimens collected in the 3rd ( $p=0.03$ ) and 12th ( $p=0.04$ ) month (Table 3). Moreover, NAG urine activity in the 12th month after transplantation correlated positively with the grade of interstitial fibrosis ( $p=0.03$ ) and tubular atrophy ( $p=0.04$ ) in specimens collected in the 12th month after transplantation. There was no correlation between urinary NAG activity and symptoms of acute rejection in the kidney allograft biopsies.

There was a significant positive correlation between NAG urine activity in the 3rd month after transplantation and the grade of proteinuria in single urine samples in the 3rd month after transplantation ( $p=0.05$ ), and a significant positive correlation between NAG urine activity in the 12th month, and the grade of proteinuria in single urine samples in the 12th month after transplantation ( $p=0.01$ ). Moreover, NAG urine activity in the 3rd month after transplantation correlated positively with the time of dialysis before transplantation ( $p=0.02$ ), and negatively with the volume of residual diuresis ( $p=0.05$ ). There was no correlation between urinary NAG activity and immunosuppressive agent concentrations. A higher NAG urine activity in the 3rd month after transplantation was observed in recipients of kidneys from donors with ischaemic or haemorrhagic stroke as a reason of death ( $p=0.03$ ).

## DISCUSSION

Introduction of calcineurin inhibitors and modern immunosuppressive agents like mycophenolate mofetil or antilymphocyte antibodies has significantly improved 1-year survival of the transplanted kidney, and reduced the occurrence of acute rejection (AR) episodes to 10–20% (Meier-Kariesche *et al.*, 2004). However, despite good 1-year outcomes, the long-term survival of renal allograft is still unsatisfactory (Kloda *et al.*, 2013). Development of IF/TA is affected by IRI, prolonged exposure to immunosuppressive drugs, viral infections, subclinical acute rejection or metabolic disorders (Cosio *et al.*, 2005; Nankivell *et al.*, 2004). Nankivell and cow-

**Table 3.** Correlation between NAG urine activity in the 3rd and 12th month after transplantation and the grade of tubular atrophy (ct) and interstitial fibrosis (ci) in graft biopsy specimens

NAG in the 3rd month	N	Spearman's R	$p$
ct 3rd month	13	0.5987014	0.03
ct 12th month	18	0.4791548	0.04
NAG in the 12th month			
ci 12th month	20	0.4784946	0.03
ct 12th month	20	0.4593998	0.04

$p$  value calculated with Spearman's Rank Order Correlation Test

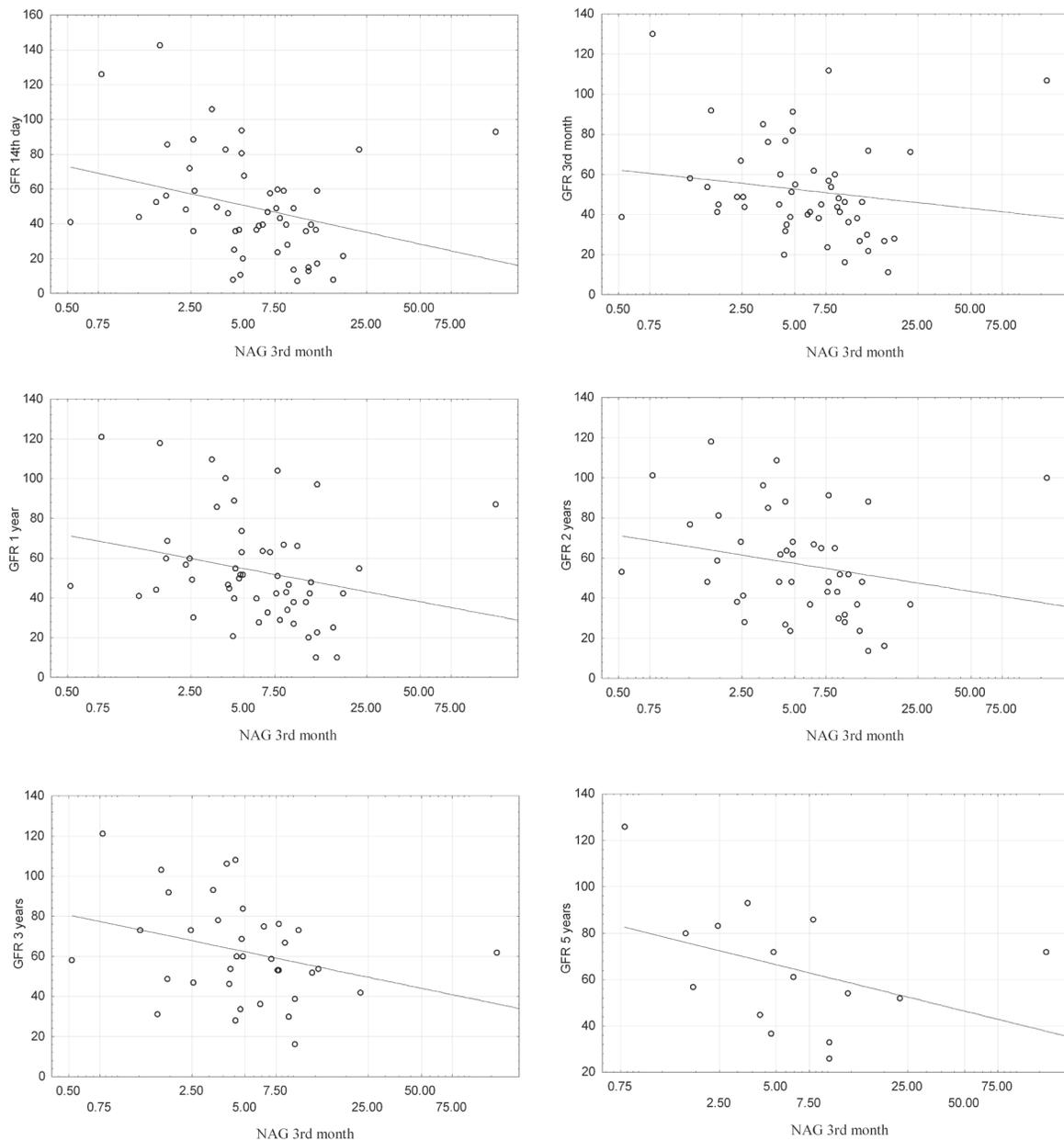


Figure 3. The correlation between urine NAG activity in the 3rd month after transplantation and GFR during study.

orkers (2003) performed prospective protocol biopsies and analysed the development of chronic damage to the transplanted kidney. The first degree of chronic allograft nephropathy (CAN) assessed with Banff classification and currently labelled as TA/IF was present in 94.2% biopsies 3 months after transplantation. This observation confirmed that early damage of tubules is associated with rapidly progressing CAN and deteriorating kidney function. Moreover, the authors observed that patients with acute tubules necrosis (ATN) are twice as likely to develop chronic allograft dysfunction.

The importance of NAG has been demonstrated in proteinuric glomerular diseases. Increased excretion of this enzyme occurred even in the absence of morphological evidence of tubular damage. One of the possibilities is that lysosomal activity of tubular cells rises when proteins filtration is higher. This mechanism might explain the correlation between urinary NAG concentration and severity of proteinuria (Bazzi *et al.*, 2002). 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 a high metabolism, and therefore, are especially jeopardized by the ischaemia (Burne-Taney *et al.*, 2005). It seems that the assessment of proximal tubule damage markers could be an efficient DGF prognostic tool. Matteucci and co-workers (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 (Kuźniar *et al.*, 2006). In our study, activity of NAG in the urine samples collected in the 3rd month after transplantation was a marker of early al-

lograft function (referred to as occurrence of DGF), as well as long-term allograft function (referred to as creatinine concentration and GFR) in the observation period of maximum 5 years. High activity of NAG correlated with deteriorating kidney function throughout the whole observation period.

For 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. We found no correlation between NAG activity and immunosuppressive agent levels, or symptoms of acute rejection in the kidney allograft biopsies. However, we observed the association of NAG activity with DGF, which results from tubule damage secondary to IRI. Doi and coworkers (2012) found an increased concentration of NAG in the urine of patients with prerenal acute kidney injury treated within intensive care units. Moreover, these authors confirmed the ischaemic hypothesis in an experiment conducted on mice, in which hypovolaemia caused an increase in urinary NAG concentration. Peto and coworkers (2005) observed IRI in dogs with closed renal arteries with subsequent reperfusion after 45 minutes. A significant increase in urinary NAG concentration was recorded. Moreover, it was proved that NAG out of all of the proximal tubule enzymes is the best prognostic kidney function marker in acute kidney injury (Herget-Rosenthal *et al.*, 2003).

In patients with glomerulonephritis, tubular atrophy is correlated with urinary NAG concentration (Holdt-Lehmann *et al.*, 2000). In our study, urinary NAG activity in the 3rd month after transplantation was associated with tubular atrophy in allograft biopsy samples collected in the 3rd and 12th month after transplantation. A similar correlation was found between urinary NAG activity in the 12th month after transplantation and the grade of tubular atrophy in kidney biopsies performed in the same month. Nauta and coworkers (2011) studied the urinary NAG levels after transplantation repeatedly, during a 3-year follow-up period. This tubular enzyme assessed in the later period after transplantation proved to be useless as an indicator of kidney function outcome. Similarly, in our study, the evaluation of NAG at time-points 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.

Proteinuria is a recognized indicator of the deteriorating transplanted kidney function (Nauta *et al.*, 2011). Concentration of urinary NAG in samples collected in the 3rd month after transplantation correlated positively with the grade of proteinuria in single urine samples collected in the 3rd and 12th month after transplantation. This is another indication that the NAG level evaluated in the early postoperative period is a prognostic marker of a long-term allograft function. Kidney tubules are damaged not only by pre- and postoperative factors, but also the health status of the donor and the recipient is important for the severity of allograft injury (Nankivell *et al.*, 2003). This was confirmed in our study, where urinary NAG activity was higher in recipients of kidneys harvested from donors with atherosclerosis, type 2 diabetes or stroke. Activity of NAG was also higher in recipients with long dialysis treatment and a small amount of residual diuresis.

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. Urinary NAG activity in samples collected in the 3rd month after transplantation is a prognostic factor of early and long-term allograft function. 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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