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CLINICAL SIGNIFICANCE OF BETA-2-MICROGLOBULIN, ENZYMES, CYTOKINES IN SERUM AND URINE IN PATIENTS WITH CHRONIC RENAL ALLOGRAFT DYSFUNCTION

ABSTRACT

The most investigations of the biomarkers of renal allograft dysfunction (RAD) are limited by early post-operational period and are aimed at diagnosis of acute rejection of renal transplant.

This work has aimed to establish additional characteristics of chronic RAD by using non-invasive biomarkers of the blood serum and urine.

MATERIALS AND METHODS. *79 patients aged 16 to 59 years (47 men and 32 women) took part in our retrospective study. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamil transferase (GGT), alkaline phosphatase (ALP), N-acetyl-β-D-glucosaminidase (NAG); interleukins (IL-2, IL-8, IL-10) and beta-2-microglobulin were evaluated.*

RESULTS. *Increased IL-10 and β2-MG serum concentration, and increased urinary concentration and activity of β2-MG, IL-2, IL-8, NAG, AP, AST, GGT were typical for chronic RAD. Only NAG was independently significantly associated with chronic RAD in multivariate regression. From the area under ROC-curves were derived, that β2-MG level in serum and urine, and the activity of NAG in urine had the excellent and good power to classify patients with satisfactory function and chronic RAD.*

CONCLUSIONS. *The increase of β2-MG in serum and urine may indicate glomerular and tubular dysfunction, respectively. An increase of urinary NAG indicates the ongoing damage of the tubules. The increase of IL-2 and IL-8 in the urine and IL-10 in serum may indicate the etiology of chronic RAD.*

KEYWORDS: *kidney transplantation; renal allograft chronic dysfunction; beta-2-microglobulin; aspartate aminotransferase; gamma-glutamil transferase; alkaline phosphatase*

The most effective method of treatment of the patients at terminal stage of chronic kidney disease is the donor kidney allotransplantation. The main problem that determines the length and quality of patient's life remains to be chronic renal allograft dysfunction (RAD) [22, 24]. There are specific (immune) and non-specific (non-immune) variants of chronic RAD that is accompanied with injuries of the nephron glomeruli or channels [24]. Predominantly these are: antigen-mediated rejection, recurrent and de novo globulomerulon nephritis and nephotoxicity of immunosupressants [22, 24] which require specific therapy and differ in terms of the prognosis. From the clinical viewpoint, apart from differential diagnosis of the variants of RAD the active (potentially curable) phase of pathological processes in the renal allotransplantat (RAT) should be distinguished from the inactive one [18].

For diagnosis of RAD the use has been made of invasive [18] and/or non-invasive [1, 32] approaches, each of them having its advantages and disadvantages. The commonly used non-invasive indicator of RAT function, serum creatinine (Cr) concentration, is the relatively less sensitive marker of the dysfunction [32] which does not evidence for pathological process activity. The non-invasive biochemical markers of injuries of both native kidneys and RAT can be the followings: enzymes

of the blood serum and urine [16, 29], low-molecular proteins [5], cytokines [14, 20] as well as specific molecules produced in the kidneys [12, 23].

Kidney disease manifests itself by the changed activity of serum enzymes [15]. At RAT pathology, as a result of pathologic glomerula filtration and inadequate reabsorption the enzymes can get into urine [16, 29]. However, issues of validation which serum or urine enzyme concentrations can give exact information about the presence of pathological process in the kidney, its localization and activity remain to be further explored. As has been noted, the preference has been given to the study of the lisosomal enzymes or their isoforms [29, 30].

Cytokines are essential mediators of pathological processes in RAT [1, 9, 14, 20]. It has been found that serum and urine interleukin (IL-2, IL-8 and IL-10) concentrations are indicative of the functional activity of various types of immune-competent cells, severity of inflammatory process at RAT and can have diagnostic and prognostic value [1, 9, 23]. At the same time opinions differ as to where one should better estimate interleukin concentrations at RAD: in the patient's peripheral blood or in the urine [9, 21].

One of the methods of laboratory kidney function diagnostic is the estimation of blood concentration and urine excretion of the low- molecular proteins such as beta-2-microglobulin (β2-MG) [12, 31]. Such estimation can reveal respectively the degree of injury of the glomerula basal membrane of the glomerules and of the epithelium of the proximal channels [7, 10, 31]. According to some authors, measurement of blood serum β2-MG concentration compared to the glomerular filtration rate (GFR) is a more accurate estimation of the blood serum β2-MG compared to GFR, especially at its low meanings, allowing assess kidney function [7, 15].

However the majority investigations of the non-invasive biomarkers of RAD are limited by early post-operational period and are aimed at diagnosis of acute rejection in renal transplant recipients [14, 16]. Only few authors studied the diagnostic value of changes in the enzyme activities [16, 30], interleukin concentrations [1, 20] and serum/urine β2-MG [12, 15] concentrations in the patients with chronic RAD. Neither has been sufficiently studied the diagnostic value of combinations of various biochemical parameters [12, 16]. The published results of investigations are rather contradictory [21, 32]. Many methods used (for instance, molecular-genetic) are expensive [14, 17] for routine clinical diagnosis.

This work has aimed to establish additional characteristics of chronic RAD by using non-invasive biomarkers of the serum and urine: enzymes (alanine aminotransferase – ALT), aspartate aminotransferase – AST), gamma-glutamil transferase – GGT, alkaline phosphatase – ALP), N-acetyl-β-D-glucosaminidase (NAG), interleukins (IL-2, IL-8, IL-10) and beta-2-microglobulin (β2-MG).

MATERIALS AND METHODS

Altogether 79 patients aged 16 to 59 years (47 men and 32 women) took part in our retrospective study. They had undergone kidney transplantation between 1997 and 2011 years at the Zaporizhzhia Regional Transplantation Centre. Average age of the patients was 38 ± 11 years. All RAT recipients included in this study received three-component immune suppressive therapy (calcinevrin inhibitor – takrolimus or cyclosporin, micofenolat mophetyl and glucocorticoids). Study materials included blood serum, urine, case histories and ambulatory charts. The patients were divided into two groups: with stable satisfactory RAT function and with its chronic dysfunction. Clinically, chronic RAD was characterized by progressive worsening of transplant function that in most cases was associated with proteinuria and arterial hypertension [22]. RAT function was estimated from blood creatinine level (Table 1) and GFR which excellently correlated between each other ($r = -0.85$, $p < 0.05$). The GFR was estimated from the Cockcroft-Golt formula (Cockcroft D. W. 1976).

The average serum creatinine level was chosen as the criterion for ascribing patients to the first or second group **(Table 1)**: 40 patients with creatinine level ≤ 150 μmol/l (1st group) and 39 patients with creatinine level ≥ 150 μmol/l (2nd group).

Serum samples for measuring biomarkers concentration were obtained after blood centrifugation taken at fasting state in the morning. Serum samples for estimating enzymes activity were studied on the same day and serum samples for measuring interleukins and β2-MG concentrations were stored in the frozen chamber at 20 °C during no longer than 3 months. Urine samples were collected at morning hours (between 7 and 9 o'clock), kept at +4 °C during three hours and centrifuged at 3000 rev/min during 15 minutes. The urine prepared this way was examined on the same day (enzymes) or stored in the freezing chamber at 20 °C during no longer than 3 months (interleukins and β2-MG) [2, 7].

Activities of the enzymes alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), gamma-glutamil transferase (GGT, EC 2.3.2.2) and alkaline phosphatase (ALP, 3.1.3.1)

Table 1. Clinical data of the patients.

PARAMETER (MEASUREMENT UNIT)		SATISFACTORY RAT FUNCTION	CHRONIC RAD	P
Age (years) [1]		37±12	40±11	0.370
Post-transplantation period, months [1]		69.1±43.3	102.5±58.7	0.062
Sex	Men [2]	21 (52.5%)	26 (66.6%)	0.190
	Women [2]	19 (47.5%)	13 (33.3%)	0.190
Calcinevrin inhibitor	Cyclosporin A[2]	34 (85%)	29 (74.4%)	0.230
	Tacrolimus [2]	6 (15%)	10 (25.6%)	0.230
Blood creatinine (μmol/l) [3]		110.5 (97.5-130.5)	195 (174-234)	0.001
Blood urea (mmol /l) [3]		6.8 (5.8-8.1)	11.5 (10.1-14.0)	0.001
GFR (ml/min) [3]		65.7±16.7	35.5±10.7	0.007
Proteinuria/CR (g/l/mmol/l) [3]		0.002 (0-0.006)	0,011 (0.005-0.022)	0.001
Specific urine density [3]		1012 (1009-1015)	1005 (1003-1010)	0.001
Arterial blood pressure	Systolic (mm Hg) [3]	130 (120-140)	150 (140-160)	0.001
	Diastolic (mm Hg) [3]	90 (80-95)	100 (90-100)	0.001

Notes:
[1] – M ± SD;
[2] – number of patients;
[3] – median (inter-quarter range).

in the serum and urine samples were studied by the biochemical method using standard kits of reagents (Filisit-Diagnostika). Optical density was measured on the spectrophotometer *"APEL"* (Japan). The serum N-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30) activity was not estimated because, according to literature data, NAG is ruined quickly and its activity in the blood is very low [2, 29]. NAG activity in the urine portions was estimated by the colorimetric method of A. A. Pokrovsky, et al. (1971) adapted by L. Ya. Migal, et al. [2] based on the p-nitrophenol amount that was formed in the course of its reaction with the substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide (*Sigma*, USA) [2].

IL-2, IL-8, IL-10 and β2-MG concentrations in the serum and urine samples were measured using the solid-phase immune-enzymic analysis. For measuring of interleukins we used the standard kits of reagents (*Vektor-Best*, Russia). The β2-MG concentration was estimated using the kits of reagents (*Orgentec Gmb*, Germany).

The obtained results on the activity of enzymes, urinary concentrations of interleukins and β2-MG were re-estimated on the creatinine (CR) level assessed in the same urine portion [14, 16, 30] by means of the Jaffe-Popper method with picric acid .

The normally distributed data were expressed by the mean value (M) and standard deviation (SD); for comparison of the results the Student t-test was used, and for assessment of the relationship between them the Pirson correlation coefficient (r) was used. The abnormally distributed data were expressed as medians and inter-quarter range; the significance of differences between the groups was assessed using the Mann-Whitney U-test; and for study of the interrelation between them the Spirman correlation coefficient (r) was used. Chronic RAT dysfunction based on the biomarkers concentration was detected using the method of monovariant and multivariant logistic regression. For estimation of the borderline levels of the activity of enzymes, interleukines and β2-MG concentrations and their diagnostic sensitivity and specificity we used the analysis of the curves of the operational characteristics (ROC-analysis). The area under the curve, its standard deviation and 95-percent statistically significant interval were assessed. The value of the indicator, at which the maximal number of patients was properly classified, served the borderline level for positive and negative results of the test.

The concentration of biomarkers marking the borderline level for decision-making was used to estimate indices of diagnostic informativeness of test accuracy [8, 26].

Statistical analysis was performed using Statistica 7.0 Program *(StatSoft Inc*., USA). To construct ROC-curve and to estimate its parameters, the SPSS 15.0 Program (*IBM*, USA) was used. The level $p < 0.05$ was assumed as statistically significant.

All investigations were performed in keeping with the principles of the European Board Convention about the human rights and biomedicine.

RESULTS AND DISCUSSION

The study groups of patients did not statistically differ by sex, age, post-transplantation term and type of immune suppressive therapy **(Table 1)**.

At the same time, the inter-group differences between main laboratory indices of RAT function were highly statistically significant **(Table 1)** evidencing in the favor of proper division of the patients into groups. Moreover, blood serum creatinine versus GFR concentration showed better correlations with other RAT function indices, such as urine concentration (0.84 against r = – 0.77) and proteinuria (r = 0.62 against r =- 0.55), in all cases $p < 0.05$.

No statistical difference between study groups was found in the activity of any blood serum enzymes **(Table 2)**. No correlation between blood serum enzyme activities and RAT function indices was found either.

In the group of patients with chronic RAD such enzymes as ALP, GGT, AST and NAG in the urine significantly exceeded the enzymuria indices in the patients with satisfactory RAT function **(Table 2)**. Notably, there was no correlation between activity of each enzyme in the serum and in the urine. Since, according to literature data [21], the main source of above enzymes in the urine is the epithelium of the proximal channels of the nephron, we consider that enzymuria analysis can provide information about its injury level. Increase of the urine ALP and GGT activities may evidence about damage of brush border of the epithelium [7, 29] where these enzymes are predominantly localized and thus accompany all stages of injury of the epithelial cell. In contrast to ALP and GGT other two enzymes, NAG and AST, are localized intracellularly, in the lysosomes and mitochondria respectively [2, 21, 29, 30]. That is why increased activity of these enzymes in the urine is the sign of deep injury of the epithelial cells of the proximal channels of the nephron.

Indices of the activity of most enzymes in the urine correlated among themselves. Since the difference in the NAG activity between the compared groups was statistically greatest **(Table 2)**, we paid special attention to this enzyme. Correlation analysis allowed establish that NAG activity was associated with the activity of all other enzymes in the urine: AST ($r = 0.47$, $p < 0.05$), ALT ($r = 0.56$, $p < 0.05$), GGT ($r = 0.56$, $p < 0.05$) and ALP ($r=0.67$, $p < 0.05$). In our opinion, these findings evidence about deep injury of the epithelium in the proximal kidney channels – dystrophy, necrobiosis and even channel necrosis [30].

In the patients with RAD the IL-2 concentration was significantly higher compared to control group. Having in mind the known IL-2 functions as mediator of T-lymphocyte activation [11, 14, 19], we assume that the reason of RAT function worsening in some patients was the T-mediated rejection [1]. According to the existing concepts about the pathogenesis of type 1 T-cellular-mediated rejection [11], the recipient

Table 2.
Enzyme activities and β2-MG concentration in blood serum of RAT recipients and their correlation with urine creatinine.

MARKER	SATISFACTORY RAT FUNCTION	CHRONIC RAD	P
AST, μM/(hour×ml) [1]	0.40 (0.20-0.40)	0.30 (0.20-0.50)	0.90
AST / Cr, μM / (hour×ml) / mM/l [2]	0.009 (0.004-0.019)	0.021 (0.011-0.033)	0.01
ALT, μM/(hour×ml) [1]	0.40 (0.30-0.50)	0.40 (0.30-0.50)	0.94
ALT / Cr, μM/(hour×ml) / mM/l [2]	0.02 (0.01-0.03)	0.01 (0.00-0.02)	0.68
GGT, mM /(hour×l) [1]	0.9 (0.7-1.4)	1.2 (0.9-1.5)	0.37
GGT / Cr, mM/(hour×ml) / mM/l [2]	0.05 (0.04-0.09)	0.08 (0.05-0.14)	0.04
ALP, nM/(sec×l) [1]	1743 (1328-2656)	2241 (954-3569)	0.96
ALP / Cr, nM/(sec×l) / mM/l [1]	91.6 (51.5-158.2)	127.9 (72.0-250.1)	0.04
NAG / Cr, nM/(sec×l) / mM/l [2]	14.3 (6.3-24.0)	21.7 (12.8-39.8)	0.01
β_2-MG, μg/ml [1]	6.48 (5.36-7.76)	8.68 (7.89-11.73)	0.01
β_2-MG / Cr, μg/ml / mM/l [1]	0.025 (0.006-0.120)	0.120 (0.043-0.194)	0.01

Notes:
[1] – Median (inter-quarter range);
[2] – M ± SD.

T-lymphocytes are capable to penetrate into the channels, discern donor HLA-antigens on the epithelial cells, get activated and release IL-2 [9]. The absence of any changes in the blood serum IL-2 concentration support this hypothesis **(Table 3)**.

K In the patients with RAD, the blood serum IL-10 concentration was significantly higher compared with satisfactory RAT function **(Table 3)**, whereas its urine concentration value did not differ significantly **(Table 3)**. IL-10 is the cytokine of type 2 lymphocytes-helpers and its effects promote clonal expansion of B lymphocytes in the lymphoid tissue and their synthesis of immunoglobulines [11, 19, 22, 24]. As known, in this era of kidney transplantation the chronic antibody-mediated rejection is the main reason of chronic RAD [13] and, probably, this precisely variant of dysfunction evidences for increasing serum IL-10 concentration.

Urine IL-8 concentration was significantly higher in the patients with chronic RAD **(Table 3)** while it was not detected in the blood serum. The source of urine IL-8 can be both local cell elements (vascular endothelium, epithelial cells and fibroblasts) and the hematopoietic cells (monocytes and lymphocytes) [23]. The published data show that highly probable reason of increased urine ІЛ-8 concentration is the inflammatory processes in the kidney of bacterial or viral etiology [27].

Table 3.
Serum interleukin concentrations and urine interleukins/ creatinine ratios in the RAT recipients, median (inter-quarter range).

MARKER	SATISFACTORY RAT FUNCTION	CHRONIC RAD	P
IL-2, pg/ml	0.20 (0.17-1.28)	0.21 (0.18-2.13)	0.10
IL-2 / Cr, pg/ml / mM/l [1]	0.18 (0.13-0.21)	0.36 (0.13-1.16)	0.04
IL-8, pg/ml	2.47 (1.67-4.39)	2.57 (2.10-4.95)	0.70
IL-8 / Cr, pg/ml / mM/l [1]	0.33 (0.10-0.60)	0.76 (0.34-3.31)	0.02
IL-10, pg/ml	2.85 (1.90-3.67)	3.73 (2.58-4.62)	0.01
IL-10 / Cr, pg/ml / mM/l [1]	0.07 (0.07-0.10)	0.09 (0.07-0.22)	0.29

Note: [1] – The data are presented in re-estimation for creatinine concentration in the same urine portion.

Table 4.
Predicting the probability of chronic RAD based on biomarkers concentration using logistic regression.

MARKER	MONOVARITE ANALYSIS			MULTIVARITE ANALYSIS		
	OR	95% CONFIDENCE INTERVAL	P	OR	95% CONFIDENCE INTERVAL	P
NAG / Cr, nM/(sec×l) / mM/l	1.26	1.07-1.49	0.006	4.13	1.21-14.09	0.023
ALP / Cr, nM/(sec×l) / mM/l	1.13	1.01-1.26	0.040	0.83	0.53-1.29	0.399
AST / Cr, μM/(hour×ml) / mM/l	1.53	0.50-4.71	0.460	-	-	-
GGT / Cr, mM/(hour×ml) / mM/l	1.11	0.76-1.61	0.580	-	-	-
IL-8 / Cr, pg/ml / mM/l	1.13	0.89-1.44	0.290	-	-	-
IL-10, pg/ml	1.63	1.01-2.26	0.040	7.68	0.49-19.67	0.146
IL-2 / Cr, pg/ml / mM/l	0.63	0.22-1.83	0.052	-	-	-
β_2-MG, μg/ml	1.99	1.20-3.31	0.006	1.38	0.68-2.79	0.373
β_2-MG / Cr, μg/ml / mM/l	1.10	1.01-1.19	0.026	1.00	0.74-1.36	1.000

Notes: NAG – N-cetyl-β-D-glucosaminidase, GGT γ-gamma-glutamyl transferase, ALP – alkaline phosphatase, AST – aminotransferase, β2-MG – β2-microglobulin, IL – interleukins, Cr -creatinine, OR – odds ratio.

Table 5.
Results of ROC- analysis.

MARKER	AREA UNDER CURVE (AUC), ± STANDARD ERROR	95%-STATISTICALLY SIGNIFICANT INTERVAL
GGT / Cr, mM/(hour ml) / mM/l	0.546 ± 0.079	0.392 - 0.701
AST / Cr, μM/(hour ml) / mM/l	0.636 ± 0.075	0.490 - 0.783
ALP / Cr, nM/(sec l) / mM/l	0.654 ± 0.069*	0.519 - 0.789
NAG / Cr, nM/(sec l) / mM/l	0.701 ± 0.061*	0.581 - 0.821
IL-2 / Cr, pg/ml / mM/l	0.643 ± 0.104	0.438 - 0.848
IL-8 / Cr, pg/ml / mM/l	0.627 ± 0.103	0.425 - 0.830
IL-10, pg/ml	0.684 ± 0.073*	0.573 - 0.823
β2-MG, μg/ml	0.858 ± 0.061*	0.738 - 0.977
β2-MG / Cr, μg/ml / mM/l	0.733 ± 0.079*	0.577 - 0.888

Notes:
NAG – N-cetyl-β-D-glucosaminidase, ALP – alkaline phosphatase, AST – aspartate amine transferase, β2-MG – β2-microglobulin, IL – interleukins, Cr -creatinine.
** – The area under the curve is statistically greater 0.5.*

Urine IL-2 and IL-8 concentrations were interrelated (r = 0.49, p<0.05). This finding may indicate co-existence of several mechanisms of RAT damage in one recipient. Rather, the cause of chronic RAD in this case is combination of pyelonephritis and acute T-cell-mediated rejection described in the literature [11, 13] that has been showed in our previous investigations [3]. It is noteworthy that the primary is pyelonephritis that causes increase of allograft antigenicity provoking rejection.

In the patient group with chronic RAD, the blood serum and urine β2-MG concentrations were significantly higher **(Table 2)**. Serum β2-MG concentration statistically correlated to lesser degree with serum creatinine concentration (r = 0.51 p < 0.05), urine β2-MG concentration (r = 0.53, p<0.05), with GFR (r = – 0.44, p < 0.05), proteinuri (r = 0.50, p < 0.05) and urine density (r = – 0.41, p<0.05) allowing explain its increase by glomerular RAT pathology [5] at which the filtration process undergoes changes. At the same time, very weak correlation between β2-MG and creatinine allows assume that another cause of increased blood serum β2-MG concentration in the patients with chronic RAD was the increase of its extrarenal synthesis that was found earlier by other authors [31].

Following kidney transplantation an increased serum β2-MG concentration may serve as the marker of acute rejection, ischemic RAT injury, cytomegaloviral infection and lympho-prolipherative disease [15]. Correlation analysis has shown interdependence between blood serum β2-MG and IL-10 concentrations (r = 0.56, p < 0.05). In view of the literature data showing that β2-MG and IL-10 are secreted by the lymphocytes in the peripheral blood [1, 5, 11, 19, 31], it is possible to assume that the cause of chronic RAD in this case is antibody-mediated rejection.

The cause of increase of β2-MG content in the urine can be both glomerular pathology and damage of renal channels at acute T-cell-mediated rejection [25], acute bacterial [28] or toxic nephritis [15] and chronic transplantation-caused nephropathy [5]. Urine β2-MG concentration slightly correlated with its blood serum concentration (r = 0.37, p < 0.05). At the same time the concentration of β2-MG was correlated with IL-8 (r = 0.45, p < 0.05), IL-2 (r =0 .72, p < 0.05), NAG (r = 0.62, p < 0.05), AST (r = 0.44, p < 0.05) and ALP (r = 0.49, p < 0.05) in urine that evidenced for damage of proximal channels of the allotransplant at which intracellular enzymes and locally synthesized cytokines enter urine and both suffer reabsorbtion. These observations indicate that the cause of increased urine β2-MG concentration is primarily the renal tubular-interstitial pathology.

Thus, urine IL-2, IL-8, β2-MG, NAG and AST concentrations and serum β2-MG and IL-10 allow to differentiate the patients with chronic RAD and satisfactory RAT function.

To assess chronic RAD predicators, the logistic regression analysis was used at the next stage of our study **(Table 4)**.

It has been established in the mono-variant model that the risk of chronic RAD development was probably related with urinary ALP and NAG activities, blood serum IL-10 concentration and blood serum and urinary β2-MG concentrations.

Only the biomarkers demonstrating statistically significant influence in the monovariant model were included to the multi-variant model. However, it was only NAG that had such influence on the probability of development of chronic RAD. It is known that epithelium of kidney channels is damaged at all forms of RAT pathology: either primarily, for instance, under influence of nephrotoxic medicines or, secondarily, for example, at glomerular or vascular pathology [7, 13, 22, 24]. Therefore it is quite natural that urinary NAG activity in large measure reflects RAT sufferings as it is resultant of all allograft injuring processes.

Predictive accuracy of the model at individual level was estimated by means of ROC-analysis **(Figs 1, 2, 3; Table 5)**. Estimated were the meanings of only those biomarkers whose concentrations differed statistically among the groups.

Indices of the area under ROC-curves indicated excellent (> 0.8) and good (> 0.7) discriminate capacity, blood serum and urinary β2-MG concentrations **(Figures 1 and 2)** and urine NAG activity **(Figure 3)**.

Figure 1. The curve for operational characteristics of blood serum β2-MG concentration.

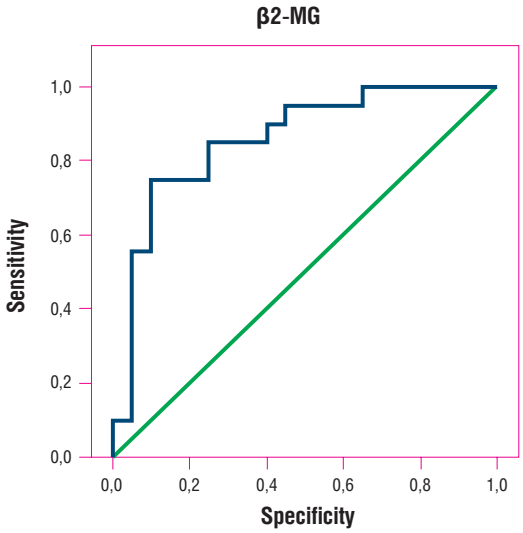


Figure 2. The curve for operational characteristics of blood serum urinary β2-MG concentration.

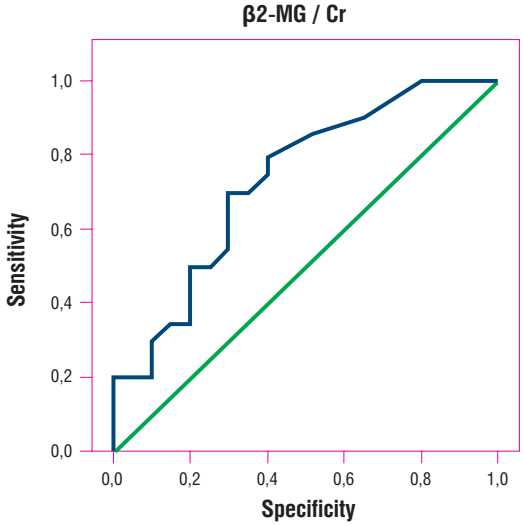
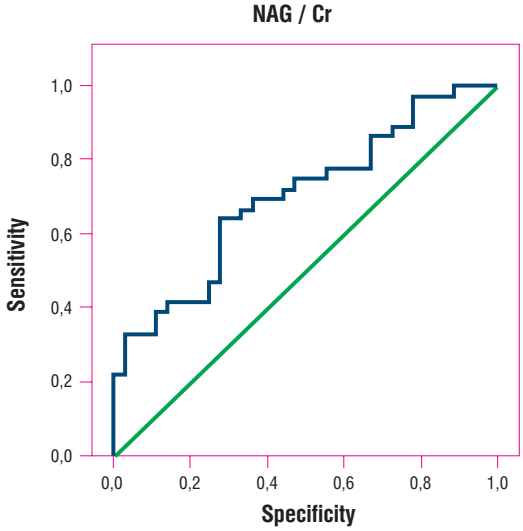


Figure 3. The curve for operational characteristics of NAG activity.



Thus, these biomarkers allow properly classify patients with satisfactory function and chronic dysfunction of the RAT. Urinary ALP activity and blood serum IL-10 concentration have median but statistically significant discriminate capacity ($p < 0.05$) **(Table 5)**.

In order to assess the possibilities of using the data of the tests for obtaining additional characteristics of chronic RAD for each indicator we have chosen the borderline level at which the test will have maximal specificity in terms of identification of patients with chronic RAD. With the use of selected borderline levels for approval of the decision, we estimated the indicators of diagnostic informativeness of the biomarkers **(Table 6)**.

The foreseeable value of positive result (FVPR) **(Table 6)** indicates that positive results of the tests for blood serum β2-MG and IL-10 and urinary NAG and IL-8 allow with high probability (> 80%) confirm the presence of chronic RAD.

The trustworthiness ratio values indicate that at the selected borderline levels the results of blood serum β2-MG and urinary NAG are most useful (PRTR ≥10); urinary IL-8 are useful (5 < PRTR < 10); and serum IL-10 (2 < PRTR ≤ 5) and urinary IL -2, β2-MG , ALP, AST and GGT are potentially useful.

The negative result of only one test – blood serum β2-MG – can be considered as useful (0.2 < NRTR ≤ 0.5) for exclusion of chronic RAD [8].

Taken together, the results indicate that by assessing urinary enzyme activity and urinary interleukin and β2-MG concentrations and blood serum interleukin and β2-MG concentrations in the patients with chronic RAD it is possible to obtain additional information about the nature, localization and activity of the pathological process.

For instance, unlike the rise of blood serum creatinine concentration, the rise of blood serum and urinary β2-MG concentrations, although it does not indicate any pathological process activity, can evidence for prevalent damage of the glomerules (rise of serum β2-MG) or of the nephron channels (rise of urinary β2-MG).

Enzymuria and primarily increase of urinary NAG activity indicates: firstly, active phase of injury process, secondly, active phase of injury process and, thirdly, domination of injury over atrophy processes [6]. Probably, urinary NAG activity is the integral measure of RAT injury under influence of factors of various genesis and mechanisms of action.

Increase of urinary and blood serum interleukin concentrations can indicate the pathological process etiology. In particular, increased urinary IL-2 concentration can indicate acute T-cell-mediated rejection. High urinary IL-8 concentration can indicate nephritis of bacterial or viral origin. In our opinion, increase of blood serum IL-10 concentration is possible at chronic active antibody-mediated rejection [4].

Table 6. Diagnostic accuracy of the markers.

MARKER, BORDERLINE LEVEL	DSens	DSpec	PPA	NPA	DEff	LRPR	LRNR
GGT / Cr > 0,195, mM/(hour×ml) / mM/l	15%	92%	67%	53%	55%	2.11	0.92
AST / Cr > 0,044, μM/(hour×ml) / mM/l	18%	92%	71%	53%	55%	2.54	0.88
ALP / Cr > 234,6, nM/(sec×l) / mM/l	26%	91%	73%	58%	60%	3.02	0.81
NAG / Cr > 34, nM/(sec×l) / mM/l	30%	97%	92%	58%	64%	10.00	0.71
IL-2 / Cr > 0,89, pg/ml / mM/l	25%	93%	80%	54%	58%	3.73	0.75
IL-8 / Cr >1,51, pg/ml / mM/l	42%	92%	85%	61%	68%	5.92	0.62
IL-10 >4,45, pg/ml	31%	91%	82%	52%	58%	3.73	0.75
β_2-MG > 8,55, μg/ml	55%	95%	92%	68%	75%	11.00	0.47
β_2-MG / Cr > 0,18, μg/ml / mM/l	30%	90%	75%	56%	60%	3.00	0.78

Notes: DSens – diagnostic sensitivity; DSpec – diagnostic specificity; PPA – positive predictive ability; NPA – negative predictive ability; DEff – diagnostic effectiveness; LRPR – likelihood ratio positive result; LRNR – likelihood ratio negative result.

CONCLUSIONS

1. ***The markers of chronic RAD are: the increase of blood serum IL-10 and β2-MG concentrations and urine β2-MG and IL-8 concentrations and the increase of NAG, ALP, AST and GGT activities.***
2. ***Enzymuria is the marker of active phase of damage of epithelium of the proximal section of the nephron.***
3. ***Increased blood serum β2-MG concentration can serve the marker of glomerular dysfunction and an increased urine β2-MG concentration can serve the marker of tubular dysfunction.***
4. ***Urine IL-2 and IL-8 and serum IL-10 concentration in the patients with chronic RAD can evidence for its etiology.***
5. ***For assessment of the course of chronic RAD, the positive results of serum β2-MG and urine NAG are most useful; the positive result of urinary IL-8 test and the negative result of serum β2-MG test are useful.***
6. ***Use of biomarkers in the combination can increase diagnostic value of each of them separately.***

REFERENCES

1. *Zograbian R, Driyanska VE, Drannik GM, et al.* Pro-(γ-IF) ta protyzapal'ni (IL-10, TFR-β) cytokiny ta i'h spivvidnoshennja jak dodatkovi oznaky dlja dyferencijnoi' diagnostyky stanu nyrkovogo alotransplantatu u viddalenomu pisljaoperacijnomu periodi. [Pro- (γ-IF) and anti-inflammatory (IL-10, TGF-β) cytokines and their correlation as additional features for differential diagnosis of renal allograft status distant postoperative period]. Ukrai'ns'kyj zhurnal nefrologii' ta dializu – Ukrainian Journal of Nephrology and Dialysis. 2007; **4(16)**:62 – 66.
2. *Mygal LYa, Bagdasarova IV, Daschenko OO.* Sposib diagnostyky stupenja aktyvnosti pijelonefrytychnogo procesu u ditej, hvoryh na pijelonefryt [A method of diagnosis of pyelonephritis in children]. Pat. na vynahid N 82170 UA, MPK (2006), G 01 N33/48. N a 2007 03509, opubl. 11.03.2008. Bjul. N 5 – Patent N 82170 UA MPK (2006) G 01 N33/48. N a 2007 03509, publ. 11.03.2008. Bull. N 5.
3. *Nikonenko AS.* Morfologicheskij analiz prichin pozdnej disfunkcii pochechnogo transplantata. [Morphological analysis of the causes of late renal transplant dysfunction]. Transplantologija – Transplantology. 2007; **9(1)**:185-187.
4. *Nikonenko AS, Trailin AV, Pleten MV, et al.* Citokiny syvorotki krovi i mochi kak markery hronicheskoj disfunkcii pochechnogo allotransplantata. Suchasni medichni tehnologiï [Cytokines of serum and urine as markers of chronic renal allograft dysfunction]. Suchasni medichni tehnologiï – Modern medical technology. 2013; **4(20)**:69-73.
5. *Pilotovich VS, Serdyuchenko NS, Arinchin VN, et al.* Beta-2-mikroglobulin i ego rol' v diagnostike nefrologicheskih zabolevanij [The role of beta-2-microglobulin in the diagnosis of nephrological diseases]. Medicinskij zhurnal – Medical Journal. 2011; **1**:80-83.
6. *Pleten MV, Trailin AV, Yefimenko NF, et al.* Diagnosticheskoe znachenie fermentemii i fermenturii u recipientov s hronicheskoj disfunkciej pochechnogo allotransplantata [Diagnostic value of enzymes in the blood and urine of recipients with chronic renal allograft dysfunction]. Eksperymental'na ta klinichna fiziologija i biohimija – Experimental and clinical physiology. 2013; **2**:90-96.
7. *Emanuel VL.* Laboratornaja diagnostika zabolevanij pochek [Laboratory diagnosis of kidney disease]. Izd. 2-e, ispr. i dop. SPb. Tver', OOO "Izdatel'stvo "Triada" – 2nd Ed., rev. and add. SPb. Tver, LLC "Publisher "Triad", 2006; 248 p.
8. Guidelines for Immunologic Laboratory Testing in the Rheumatic Diseases: An Introduction Arthritis & Rheumatism (Arthritis Care & Research). American college of rheumatology ad hoc committee on immunologic testing guidelines. 2002; **47(4)**:429-433.
9. *Amirzargar A, Lessan-Pezeshki M, Fathi A, et al.* TH1/TH2 cytokine analysis in Iranian renal transplant recipients. Transplant. Proc. 2005; **37(7)**:2985-2987.
10. Cieciura T, Urbanowicz A, Perkowska-Ptasinska A, et al. Tubular and glomerular proteinuria in diagnosing chronic allograft nephropathy with relevance to the degree of urinary albumin excretion Transplant Proc. 2005; **37(2)**:987-990.
11. *Cornell LR, Smith N, Colvin RB, et al.* Kidney Transplantation: Mechanisms of Rejection Acceptance Annual Review of Pathology. 2008; **3**:189-220.
12. *Dieterle F, Perentes E, Cordier A, et al.* Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury. Nat. Biotechnol. 2010; **28(5)**:463-469.
13. *El-Zoghby ZM, Stegall MD, Lager DJ, et al.* Identifying specific causes of kidney allograft loss Am. J. Transplant. 2009; **9(3)**:527-535.
14. *Gupta RK, Jain M, Sharma RK.* Serum & urinary IL-2 levels as predictors in acute renal allograft rejection. Indian J. Med. Res. 2004; **119(1)**:24-27.
15. *Keown P A.* Predicting long-term outcome in renal transplantation. Kidney International. 2013; **84**:650-652.
16. *Lisowska-Myjak B.* Serum and Urinary Biomarkers of Acute Kidney Injury. Blood Purif. 2010; **29**:357-365.
17. *McDaniel DO, Rigney DA, McDaniel KY, et al.* Early expression profile of inflammatory markers and kidney allograft status. Transplant. Proc. 2013; **45(4)**:1520 – 1523.
18. *Mengel M, Gwinner W, Schwarz A, et al.* Infiltrates in protocol biopsies from renal allografts Am. J. Transplant. 2007; **7**:356 – 365.
19. *Nankivell BJ.* Rejection of the Kidney Allograft. N. Engl. J. Med. 2010; **363**:1451-1462.
20. *Omrani MD, Mokhtari MR, Bagheri M, et al.* Association of interleukin-10, interferon-gamma, transforming growth factor-beta, and tumor necrosis factor-alpha gene polymorphisms with long-term kidney allograft survival. Iran. J. Kidney Dis. 2010; **4(2)**:141-146.
21. *Ozer JS, Dieterle F, Troth S, et al.* A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. Nat. Biotechnol. 2010; **28**:486-494.
22. *Pascual J, Pérez-Sáez MJ, Mir M, et al.* Chronic renal allograft injury: early detection, accurate diagnosis and management. Transplant Rev (Orlando). 2012; **26(4)**:280-290.
23. *Pereira AB, Teixeira AL, Rezende NA, et al.* Urinary chemokines and anti-inflammatory molecules in renal transplanted patients as potential biomarkers of graft function: a prospective study. Int. Urol. Nephrol. 2012; **44(5)**:1539-1548.
24. *Sellarés J, de Freitas DG, Mengel M, et al.* Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am. J. Transplant. 2012; **12(2)**:388-399.
25. *Schaub S, Mayr M, Hönger G, et al.* Detection of subclinical tubular injury after renal transplantation: comparison of urine protein analysis with allograft histopathology. Transplantation. 2007; **84(1)**:104-112.
26. *Schoenbach VJ, Rosamond WD.* Understanding the Fundamentals of Epidemiology: an Evolving Text. The phenomenon of disease. Chapel Hill. 2001; Chapter **4**:59-81.
27. *Sheu JN, Chen SM, Meng MH.* The role of serum and urine interleukin-8 on acute pyelonephritis and subsequent renal scarring in children. Pediatr. Infect. Dis. J. 2009; **28(10)**:885-890.
28. *Sekine H, Kawasaki Y, Ohara S, et al.* Focal bacterial nephritis without pyuria in a boy presenting with high urinary β2-MG and NAG levels. Fukushima J. Med. Sci. 2014; **60(1)**:91-94.
29. *Skálová S.* The diagnostic role of urinary N-acetyl-beta-D-glucosaminidase (NAG) activity in the detection of renal tubular impairment. Acta Medica. 2005; **48**:75-80.
30. *Westhuyzen J, Endre ZH, Reece G, et al.* Measurement of tubular enzymuria facilitates early detection of acute renal impairment in the intensive care unit. Nephrol. Dial. Transplant. 2003; **18**:543-551.
31. *Woo J, Floyd M, Cannon D C.* Albumin and beta2-Microglobulin Radioimmunoassays Applied to Monitoring of Renal-Allograft Function and in Differentiating Glomerular and Tubular Diseases. Lin. Chem. 1981; **27(5)**:709-713.
32. *Wu I, Parikh CR.* Screening for kidney diseases: Older measures versus novel biomarkers. Clin. J. Am. Soc. Nephrol. 2008; **3**:1895-1901.

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