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NGAL AND CYSTATİN C: TWO POSSİBLE EARLY MARKERS OF DİABETİC NEPHROPATHY İN PATİENTS WİTH TYPE 2 DİABETES MELLİTUS

NGAL И CYSTATİNC ДВА МОЖНИ РАНИ МАРКЕРИ ЗА ДИЈАБЕТИЧНА НЕФРОПАТИЈА КАЈ ПАЦИЕНТИ СО ДИЈАГНОСТИЦИРАН ДИЈАБЕТЕС МЕЛИТУС ТИП2

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Abstract

Introduction. Diabetic nephropathy (DN) is a progressive renal impairment characterized by impaired renal architecture and function and is one of the leading causes of permanent renal impairment. Patients with DN have a high mortality rate, which is primarily due to cardiovascular complications. In everyday practice in the Republic of North Macedonia, serum creatinine, microalbuminuria and glomerular filtration rate are used to detect DN. However, these standard tests do not always allow for detection of initial DN damage.

Aim. The aim of this study was to investigate the role of NGAL (in urine) and Cystatin C (in serum) values as adjunctive testing of existing markers (microalbuminuria and creatinine) in unmasking early structural and functional renal impairment in asymptomatic patients with type 2 diabetes mellitus (DM type 2).

Methods. This was a prospective, observational (6-month follow-up) study, involving 60 patients aged 35-70 years. The first two groups were patients with diagnosed DM type 2 for a minimum of 5 years, 15 patients diagnosed with DM type 2 with diabetic nephropathy and 15 patients without diabetic nephropathy. The third group consisted of healthy respondents (30). In addition to standard biochemical analyses, the three groups were also examined for body fluid concentrations of NGAL (architect urine NGAL) and Cystatin C (nephelometry), as well as standard biomarkers for renal nephropathy (serum creatinine and microalbumin).

Results. The respondents from the three analyzed groups did not differ significantly in terms of gender structure (p=0.71) and age (p=0.068). The study found that (the core values) baseline creatinine, microalbuminuria, NGAL and Cystatin C serum levels were higher in patients diagnosed with DM type 2 and diabetic nephropathy (DN) compared to those with diabetes and without diabetic nephropathy in healthy trials. Also, after 6 months of follow-up, it was proven that in patients diagnosed with DM type 2 and DN all four parameters were higher with confirmed significance unlike the group of patients with DM type 2 without DN. In the group with diabetes and diabetic nephropathy, during the re-evaluation after 6 months of monitoring we registered a non-significant increase in the biomarker NGAL p=0.16), and a significant increase in the biomarker Cystatin C (p=0.016). There was a statistically significant correlation between baseline creatinine values and baseline control values of Cystatin C (p<0.0001), creatinine and NGAL values after a 6-month re-evaluation (p=0.014), all of which were positive. The correlation between the two biomarkers NGAL and Cystatin C were statistically insignificant in the first measurements (p=0.160), and were significant and direct positive on the second measurements, after 6 months (r=0.536, p=0.039). The two markers changed in direct proportion to the serum, with the increasing of one marker in the serum. Also, the other biomarker increased, and vice versa.

Conclusion. NGAL and Cystatin C, biomarkers of renal impairment, are correlated with decreased renal function in patients with DM type 2, suggesting that NGAL and Cystatin C may be used as adjunctive tests to existing ones (creatinine and microalbuminuria) to unmask early renal dysfunction.

Keywords: diabetic nephropathy, NGAL, Cystatin C, diabetes type 2

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Апстракт

Вовед. Дијабетичната нефропатија (ДН) е прогресивно бубрежно оштетување кое се карактеризира со нарушување на бубрежната архитектура и функција и е една од водечките причини за трајно бубрежно оштетување. Пациентите со ДН имаат висок

морталитет, кој пред се се должи на кардиоваскуларни компликации. Во секојдневната пракса во Р. Северна Македонија за детекција на ДН се користат серумскиот креатинин, микроалбуминурија и гломеруларна филтрациона рата. Меѓутоа овие стандардни тестови не овозможуваат секогаш детекција на почетни оштетувања кај ДН.

Цел. Целта на оваа студија беше да се истражува улогата на вредности на NGAL (во урина) и CysC (во серум), како дополнителни-суплементарни тестирања на постоечките маркери (микроалбуминурија и креатинин) при демаскирање на рано структурно и функционално бубрежно оштетување кај асимптоматски, нормоалбуминурични пациенти со дијабетес мелитус тип 2 (ДМ тип2).

Методи. Студијата е проспективна, обсервациска (6 месечно следење), во која беа вклучени 60 пациенти, на возраст од 35-70 години. Првите две групи беа пациенти со дијагностициран ДМ тип 2 во траење од минимум 5 години (15 пациенти со дијагностициран ДМ тип 2 со дијабетична нефропатија и 15 пациенти без дијабетична нефропатија). Третата група ја сочинуваа здрави испитаници (30). Освен стандрадните биохемиски анализи, кај трите групи беа иследувани концентрации во телесните течности на NGAL (ARCHITECT Urine NGAL) и Cystatin C (нефелометрија), како и стандардните биомаркери за бубрежна нефропатија (серумски креатинин и микроалбуминурија).

Резултати. Испитаниците од трите анализирани групи не се разликуваа сигнификантно во однос на половата структура (p=0.71) и возраста (p=0.068). Студијата покажа дека базичните вредности на креатинин, микроалбуминурија, NGAL и CystatinC во серум, беа повисоки кај пациентите со дијагностициран ДМ тип 2 и дијабетична нефропатија (ДН), во споредба со тие со дијабетес и без дијабетична нефропатија и здравите испитаници. Исто така после 6 месечно следење се докажа дека кај пациентите со дијагностициран ДМ тип2 и ДН сите четири параметри беа повисоки со потврдена сигнификантност за разлика од групата на пациенти со ДМ тип2 без ДН. Во групата со дијабетес и дијабетична нефропатија, при ре-евалуацијата по 6 месечно следење регистриравме несигнификантно зголемување на биомаркерот NGAL (p=0.16), а сигнификантно зголемување на биомаркерот Cystatin C (p=0.016). Се покажа статистичка сигнификантна корелација на базичните вредности на креатинин со базичните и контролни вредности на Cystatin C (p<0.0001), и на вредностите на креатинин и NGAL по ре-евалуација на 6 месеци (p=0.014), и сите овие корелации се позитивни. Корелацијата меѓу двата биомаркери NGAL и Cystatin C беше статистички несигнификантна на првото мерење (p=0.16), а сигнификантна и директна, односно позитивна при второто мерње, по 6 месеци (r=0.536,p=0.039). Двата маркери се менуваа правопропорционално во серумот, со зголемување на едниот маркер во серумот, се зголемуваше и другиот биомаркер, и обратното.

Заклучок. NGAL и Cystatin C, биомаркери на бубрежно оштетување, се во корелација со пад на бубрежната функција кај пациенти со ДМ тип2, што укажува на тоа дека тие може да се користат како дополнителни тестови на постоечките (креатинин и микроалбуминурија) со цел да се демаскира раната бубрежна дисфункција.

Клучни зборови: дијабетична нефропатија, NGAL, Cystatin C, диајбетес тип 2

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by chronic hyperglycemia due to an inherited and/or acquired deficiency in pancreatic insulin production, or the ineffectiveness of the produced insulin [1,2]. Diabetes occurs due to the interaction of various factors, primarily genetic factors, lifestyle and environmental factors.

Diabetes is on a pandemic scale and is one of the major health problems of the 21st century. According to the IDF (International Diabetes Federation), the total number of people with diabetes in the world as of 2019 is 463 million, and this figure is expected to exceed 700 million by 2045 [3].

The most common types of diabetes are: type 1 diabetes mellitus, type 2 diabetes mellitus and gestational diabetes. Type 2 diabetes is more common and covers about 90-95% of all types of diabetes, both globally and in our country [1,2,4].

DM causes acute and chronic micro- and macrovascular complications [5-7].

Acute complications include hypoglycemia, diabetic ketoacidosis, and hyperosmolarity [5]. Chronic microvascular complications include: diabetic retinopathy, nephropathy, and neuropathy [6,7].

Whether and when chronic microvascular complications occur depends on many factors, including:

- Duration of diabetes
- Diet and physical activity
- Appropriate therapy
- Control of glycemia-HbA1c values, fasting glycemia values and postprandial glycemia

Diabetic nephropathy (DN) is a progressive renal impairment characterized by impaired renal architecture and function, and is one of the leading causes of permanent renal impairment [8-10]. As a result, these patients most often require renal replacement therapy (RRT) and/or hemodialysis (HD). In recent years, the number of patients on RRT has been continuously increasing, both in the world and in the Republic of North Macedonia. Thus, in 2002 the total number of patients on RRT was

Fig. 1. Number of patients in need of renal replacement therapy - RRT in the Republic of Macedonia for the period from 2002 to 2016; *Data from the annual registers of ERA-EDTA, *Internal data for 2012-2013, *HD-hemodialysis, *PD-peritoneal dialysis, *Tx (transplant), *Total-Total

1056, of which 92% were on HD. In 2016 the number of patients on RRT was 1665, of which 86% were on HD (Figure 1) [11].

The pathophysiological changes in DN that leads to reduction in renal function are associated with cellular and extracellular disorders in the glomerular and tubulointerstitial structures [10,12,13]. Glomerular and tubular-interstitial kidney injury plays a role in the pathogenesis of DN [13]. Patients with DN have a high mortality rate, which is primarily due to cardiovascular complications.

In everyday practice in R. of North Macedonia, serum creatinine, albuminuria and glomerular filtration rate are used to detect DN.

Microalbuminuria has generally been considered the earliest marker of the development of diabetic nephropathy, and is often associated with a significant glomerular damage. However, recent studies have shown that MA does not always reflect the present renal impairment. In addition, several lines of evidence suggest that early damage to glomerular and tubular structures may be present in subjects with normal albuminuria [13,15,16].

It is necessary to identify markers that will detect early tubular damage independently of the development of albuminuria in patients with early DN and progression, as it may play a significant role in the management of cases of renal failure that are within normal albuminuria [13,15-17].

New biomarkers such as NGAL (neutrophil gelatinase-associated lipocalin) and Cystatin C are thought to be more sensitive to the need of early DN detection.

NGAL, also known as lipocalin 2, is composed of 178 amino acids. It is a 25 kDa protein, first purified and identified in 1993 by Kjeldsen *et al*., and appears to be a promising biomarker [13,17]. It is mainly produced in the renal tubules in response to structural injury to the kidneys, but also to a lesser extent in the lungs, trachea, stomach, and colon, and excreted in the urine [18,19]. NGAL values may be affected by renal disease, hypertension, inflammatory conditions, hypoxia, and malignancies [18]. NGAL as a renal biomarker was first described in 2003 by causing experimental renal ischemia in mice [20].

Unlike conventional serum markers, such as creatinine, NGAL is considered a marker of renal structural damage, whose plasma and urine values increase as a result of tubular renal damage. Its values rise before renal damage is detected by other methods [18,19,21]. Except in acute and chronic renal failure, this biomarker has significance in the progression of renal damage. In previous studies, NGAL has been shown to be effective in the early diagnosis of acute renal injury (ACI), and in several clinical studies has also been validated for its prognostic role in cardiovascular morbidity [22-24].

Cystatin C is a small protein that is filtered by the body through the glomeruli, which has a high correlation with the degree of glomerular filtration rate (GFR) [25]. It is not affected by inflammatory conditions, muscle mass, sex, body composition and age (after the age of 12 months) [26]. The superiority of Cystatin C (CysC) over the other markers of renal impairment lies in its ability to remain unbound to proteins and to filter freely through the glomeruli. In healthy subjects, CysC is almost freely filtered by the glomeruli and is almost completely reabsorbed in the proximal tubules, as are other low molecular weight proteins with no or only partial tubular secretion.

In renal impairment, with decreasing GFR, the values of this biomarker increase. Several studies have shown that when GFR and creatinine levels are still within normal limits, CysC rises when there is initial renal impairment [27,28].

High CysC values in patients diagnosed with diabetes mellitus increase the risk of cardiovascular morbidity and progression of atherosclerosis [29,30].
Diagnosis of DN (diabetic nephropathy) in the early stages is of particular importance, because therapeutic measures taken in the early stages of DN prevent the progressive course of DN, and thus reduce cardiovascular and overall mortality in the population with diabetes [8,9].
Screening for other microvascular and macrovascular complications is also important to treat and prevent further progression of the damage.

Aims

- The aim of this study was to compare serum values of NGAL and CysC in the three groups of patients, and to determine the possible predictor roles of the serum values of these biomarkers as additional-supplementary testing of urinary albumin excretion in albuminuria, unmasking early structural and functional renal impairment in patients with type 2 diabetes mellitus (DM type 2).
- To see if there was a correlation between the serum values of the marker-NGAL and CysC in relation to serum values of creatinine and microalbuminuria.
- To determine if the values of the new biomarkers (NGAL, Cystatin C) increase with the progression of renal nephropathy.

Material and methods
Study design

This was a prospective, observational study, involving three groups of patients, a total of 60 in number, aged 35-70 years, with a follow-up period of 6 months.
Patients were divided into three groups:

1. In the first group of 15 patients diagnosed with diabetes mellitus (DM) type 2 lasting for more than 5 years, diabetic nephropathy was not detected with standard biomarkers (serum creatinine, albuminuria and glomerular filtration rate).
2. In the second group, 15 patients diagnosed with diabetes mellitus (DM) type 2 with a duration of more than 5 years, in whom with standard biomarkers (serum creatinine, albuminuria and glomerular filtration rate) renal nephropathy was not detected.
3. The third group included 30 healthy individuals (who appeared at the Clinic for screening for diabetes or other endocrine disorder, but had normal findings).

The study was performed at the University Clinic for Endocrinology-Skopje in cooperation with the PHI University Clinic for Clinical Biochemistry- Skopje.
Patient's choice was not influenced by their gender, race, ethnicity or socioeconomic status.
During the study we adhered to the criteria for Basic Good Clinical Practice, the Law on Health Care of the Republic of Macedonia and the Law on Patient's Rights of the Republic of Macedonia.
The selection of patients was performed on the basis of a signed informed consent, as well as the application of criteria for inclusion and exclusion in the study.

Criteria for inclusion and exclusion in the study:
Inclusion criteria: patients with diagnosed DM type 2, age 35-70 years, sex: m/f.
Exclusion criteria: younger than 35 and older than 70 years, patients with DM type 1, gestational diabetes, active urinary tract infection, pregnant patients, if on glucocorticoid therapy, other renal diseases, active malignancy, other chronic diseases, or if the patient requested to be withdrawn from the study.
Venous blood and urine were taken from all three groups of patients to test for standard and new biomarkers for diabetic nephropathy:

- creatinine,
- microalbuminuria,
- NGAL (NGAL- neutrophil gelatinase-associated lipocalin),
- Cystatin C

In patients diagnosed with DM type 2, the diagnosis was confirmed by determination of fasting glycemia, postprandial glycaemia, and HbA1c.
After 12 hours of fasting, morning venous blood was taken to determine creatinine, CysC, HbA1c, and fasting glycemia. Blood was collected in special tubes to determine the specific CysC marker. Part of the blood was centrifuged to separate the serum and kept at -80 until the markers were finally determined.
CysC concentration was measured by immuno-nephelometric technique using a BN Prospec nephelometer (Dade Bering, Siemens Health Diagnostics, Liederbach, Germany). Normal biomarker values are 0.62-1.11 mg/L. The inter-assay coefficient of variation (CV) for the analysis was 5.05% and 4.87% at mean concentrations of 0.97 and 1.90 mg/L, respectively.
Microalbuminuria and NGAL were determined from a urine sample.
NGAL values were measured using the ARCHITECT Urine NGAL assay, which is an immunoassay of chemiluminescent microparticles (CMIA) for quantitative determination of NGAL in urine. Urine can be stored at room temperature for up to 24 hours, or up to 7 days at a temperature of 2-8 degrees Celsius. The calibration range is 0.0 ng/mL-1500 ng/mL, while the measurement interval is 10.0-1500 ng/mL. Normal values are up to 131.7 ng/mL. The ARCHITECT Urine NGAL analysis is designed to have an inaccuracy of <10% of the total CV (coefficient of variation).
Other procedures performed in patients: medical history and status, blood count, lipid status, fasting glycemia and postprandial glycemia.
All of the above examinations and procedures were performed in the three groups of patients. In the first

two groups of patients (patients diagnosed with DM type 2 with and without diabetic nephropathy) the tests were repeated and compared to the control that was performed after 6 months.
In the first two groups (patients diagnosed with DM type 2), Doppler ultrasonography of the kidneys was performed.

Statistical analysis

Statistical processing and data analysis was performed with the statistical program SPSS for Windows, 23.0.
Kolmogorov-Smirnov and Shapiro Wilk's test were used to test the normality in data distribution.
Quantitative parameters are represented by an arithmetic mean with standard deviation and median mean, qualitative parameters are represented by absolute and relative numbers.
Bivariate analysis was performed to compare the analyzed groups (DM with LV, DM without LV and KG). Pearson Chi-square test was used to compare these groups in terms of qualitative characteristics. Analysis of Variance, Kruskal-Wallis and Mann-Whitney test were used to compare these groups in terms of quantitative characteristics. The Wilcoxon Matched pairs test was used to compare the two groups with diabetes. The correlation of the analyzed markers in the group with DM and DN was analyzed with Pearson correlation coefficient. Values of p <0.05 were taken as statistically significant.

Results

The respondents from the three analyzed groups did not differ significantly in terms of gender structure (p =0.71) and age (p=0.068). Patients in the three groups were predominantly male, with a prevalence of 53.3% in the group with diabetes and diabetic nephropathy, 66.7% in the group with diabetes without diabetic nephropathy and 55% in the group of healthy subjects. Patients with diabetes and chronic nephropathy were insignificantly older than patients in the other two groups (61.20±4.9, 55.80±8.2, 55.95±7.8, respectively) (Table 1). Comparison of baseline creatinine, microalbuminuria,

Table 1. Socio-demographic characteristics of respondents

Variable	Groups DM type 2 with DN	Groups DM type 2 without DN	KG	p value
Sex (n%)				
Female	7 (46.67)	5 (33.33)	9 (45)	X^2=0.67 p=0.71 ns
Male	8 (53.33)	10 (66.67)	11 (55)	
Age (mean±SD)	61.20±4.9	55.80±8.2	55.95±7.8	F=2.8 p=0.068 ns

X^2 (Pearson Chi-square) F (Analysis of Variance)

NGAL, and cystatin C values in serum showed that patients with diabetes and diabetic nephropathy had higher values than diabetic patients without diabetic nephropathy and healthy subjects.
For p<0.0001, a statistically significant difference in serum creatinine concentrations was confirmed between the three groups. Post-hoc analysis for intergroup comparisons showed that this overall significance was due to significantly higher creatinine in patients with DM and DN compared to patients with DM without DN (median 129.6 *vs.* 67.5; p=0.0009), and in patients with DM and DN in relation to CG patients (median 129.6 *vs.* 64; p=0.0009).
A statistically significant difference was confirmed between the three groups in relation to the initial serum values of microalbuminuria (p<0.0001), which was

Table 2. Basic values of creatinine, microalbuminuria, NGAL, Cystatin C in respondents of the three groups

Variable (basic)	Groups DM type 2 with DN	Groups DM type 2 without DN	KG	p value
Creatinine (mean±SD)	133.19±50.7	72.09±13.7	65.77±14.2	H=24.18 p=0.000 sig
median (IQR)	129.6 (112.3-147)	67.5 (63.8-80)	64 (56.1-77.4)	[a]p=0.0009 [b]p=0.000006
Microalbuminuria (mean±SD)	80.11±52.4	18.47±22.9	12.04±5.8	H=23.31 p=0.000 sig
median (IQR)	100 (40.5-100)	10.6 (10.6-15.6)	10.6 (9.9-12)	[a]p=0.0016 [b]p=0.000009
NGAL (mean±SD)	164.79±76.9	49.85±69.6	23.24±22.6	H=19.06 p=0.0001 sig
median (IQR)	166.2 (123.2-224)	20 (11.8-34)	21.85 (10.1-25.5)	[a]p=0.0033 [b]p=0.000082
CystatinC (mean±SD)	1.61±0.9	0.88±0.3	0.69±0.1	H=30.17 p=0.000 sig
median (IQR)	1.29 (1.2-1.6)	0.77 (0.74-0.92)	0.69 (0.58-0.82)	[a]p=0.0025 [b]p=0.000000

p (Kruskal-Wallis test) post-hoc Mann-Whitney test; [a]p (DM type 2 with DN *vs.* CG) [b]p (DM type 2 without DN *vs.* CG)

due to significantly higher values in the group with DM and DN compared to the group with DM without DN (p=0.0016) and in relation to healthy subjects (p= 0.000009). In the three groups were registered main values of 80.1 were, 18.5 and 12 mg/L, consequently (Table 2).
A total statistically significant difference was confirmed in the basal serum values of NGAL (p=0.0001). Post-hoc analysis for intergroup comparisons showed that patients with DM and DN had significantly higher baseline values of NGAL than patients with DM without DN (median 166 *vs.* 20; p=0.0033), and in relation to healthy subjects (median 166.2 *vs.* 21.85; p=0.00008).
The Cystatin C biomarker presented significantly different baseline values between the two groups with diabetes (p=0.0025), and between the group with DM and DN and CG (p<0.0001). The mean values of this marker were 1.29, 0.77 and 0.69 mg/L in the group with DM and DN, the group with DM without DN, and CG, consequently.
We compared patients from both groups with diabetes in terms of analyzed serum parameters after 6 months of follow-up. According to the results in Table 3, all parameters were higher in the group with diagnosed diabetic nephropathy, with confirmed significance of p= 0.0006 for creatinine, p=0.00007 for microalbuminuria, p =0.00036 for NGAL, and p=0.019 for Cystatin C. Serum creatinine concentrations of 132 and 79.8 mmol/L were measured in the group with DM and DN and the group with DM without DN after 6 months, respectively; serum values of microalbuminuria of 60 and 12 mg/L, respectively; serum NGAL values of 178 and 30.2 ng/ml, respectively; and, serum Cystatin C values of 1.48 and 0.88 mg/L, respectively (Table 3).

Table 3. Values of creatinine, microalbuminuria, NGAL and Cystatin C in respondents of the three groups – 6-month follow-up

Variable (after 6 month follow-up)	Groups DM type 2 with DN	DM type 2 with DN	P value
Creatinine (mean ± SD)	169.42±155.3	81.79±15.9	Z=3.44 p=0.0006 sig
median (IQR)	132 (96-152)	79.8 (73-90)	
Microalbuminuria (mean±SD)	109.97±85.2	23.35±24.2	Z=3.98 p=0.00007 sig
median (IQR)	60 (50-200)	12 (11-20.3)	
NGAL (mean ± SD)	175.87±75.4	57.80±71.9	Z=3.57 p=0.00036 sig
median (IQR)	178 (124-220)	30.2 (15.1-48)	
Cystatin C (mean ± SD)	1.91±1.7	126.25±331.1	Z=2.34 p=0.019 sig
median (IQR)	1.48 (1.22-1.68)	0.88 (0.8-1.39)	

p (Mann-Whitney U Test)

In the group with diabetes and diabetic nephropathy, during the re-evaluation after 6 months of monitoring we registered a non-significant increase in the biomarker NGAL (p=0.16) and a significant increase in the biomarker Cystatin C (p=0.016). Mean serum NGAL concentrations were 166.2 ng/ml on the first measurement and 178 ng/ml on the second measurement.
Mean serum Cystatin C concentrations were 1.29 mg/L on the first measurement and 1.48 mg/L on the second measurement (Table 4) (Figure 2 and 3).

Table 4. Values of NGAL and Cystatin C – group with DM and DN

Group with DM and DN		Descriptive Statistics mean±SD	median (IQR)	p value
NGAL (ng/ml)	basic	164.79±76.9	166.2(123.2-224)	Z=1.42
	re-evaluation	175.87±75.4	178(124-220)	p=0.16 ns
Cystatin C (mg/L)	basic	1.61±0.9	1.29(1.2 -1.6)	Z=2.41
	re-evaluation	1.91±1.7	1.48(1.22 -1.68)	p=0.016 sig

Z (Wilcoxon Matched pairs test)

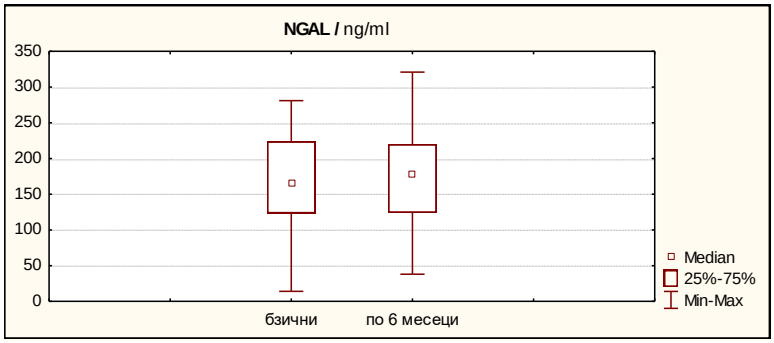

Fig. 2. Mean values of NGAL - group with DM and DN

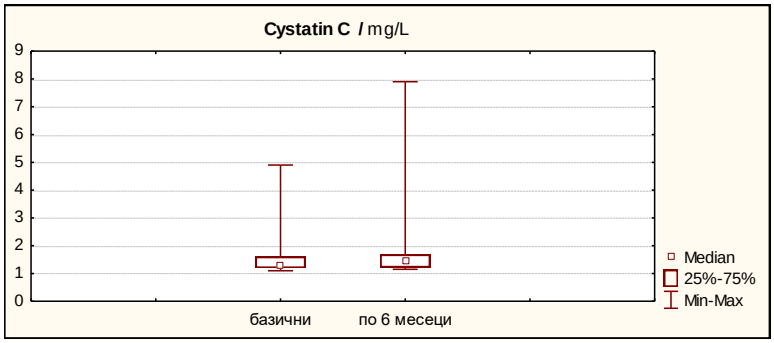

Fig. 3. Mean values of Cystatin C - group with DM and DN

The results of the study on the association of creatinine and microalbuminuria with the biomarkers NGAL and Cystatin C in the group with DM and DN showed a statistically significant correlation of baseline creatinine with baseline and control values of Cystatin C (p <0.0001) and NGAL after 6-month re-evaluation (p= 0.014). According to Pearson correlation coefficient, all these correlations were positive, i.e. direct, with increasing serum creatinine values the biomarkers NGAL and Cystatin C increased, and vice versa (r=0.617, r=0.809, r=0.879, consequently) We did not find a significant correlation of microalbuminuria with NGAL and Cystatin C at baseline and on re-evaluation (Table 5) (Figure 4, 5 and 6).

Table 5. Correlation of microalbuminuria and creatinine with NGAL and Cystatin C

Correlation	r	p-level
basic creatinine & basic NGAL	0.285	0.304 ns
re-evaluation creatinine & re-evaluation NGAL	0.617	0.014 sig
basic creatinine & basic Cystatin C	0.809	0.000 sig
re-evaluation creatinine & re-evaluation Cystatin C	0.879	0.000 sig
basic microalbuminuria & basic NGAL	0.198	0.479 ns
re-evaluation microalbuminuria & re-evaluation NGAL	0.229	0.410 ns
basic microalbuminuria & basic Cystatin C	0.233	0.402 ns
re-evaluation microalbuminuria & re-evaluation Cystatin C	0.452	0.090 ns

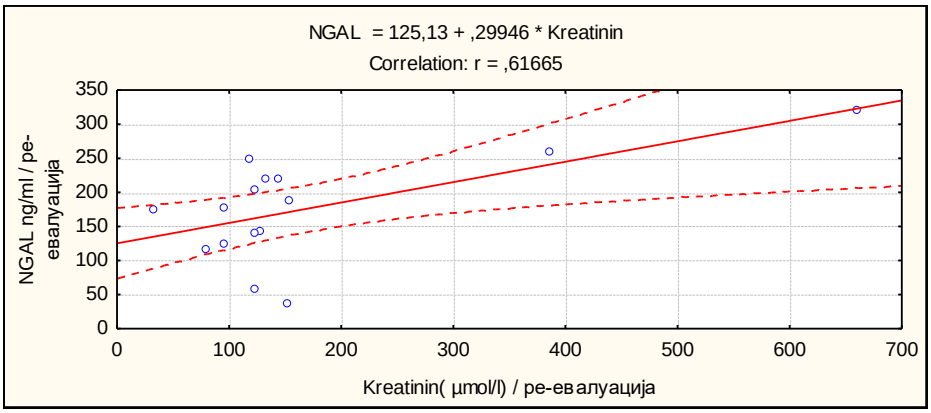

Fig. 4. Correlation of NGAL with creatinine - after 6 months

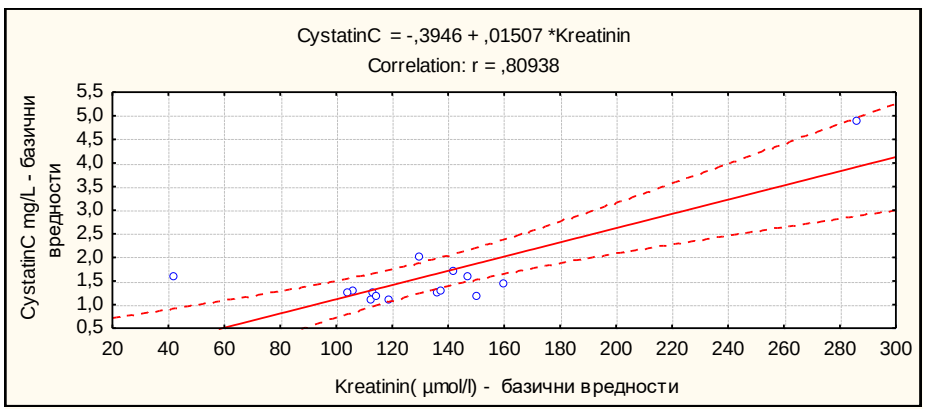

Fig. 5. Correlation of Cystatin C with creatinine - baseline values

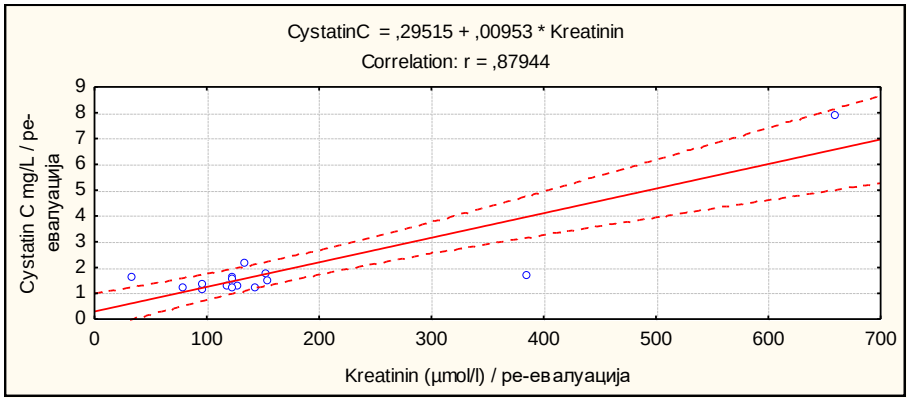

Fig. 6. Correlation of Cystatin C with creatinine - after 6 months

The correlation between the two biomarkers NGAL and Cystatin C was statistically insignificant on the first measurement (p=0.16), and significant and direct, i.e. positive on the second measurement, after 6 months (r =0.536, p=0.039). Both markers changed in direct proportion to serum, by increasing one marker in the serum, the other biomarker increased, and vice versa (Table 6) (Figure 7).

Table 6. Correlation between the two biomarkers NGAL and Cystatin C

Correlation	R	p-level
basic NGAL & basic Cystatin C	0.386	0.156 ns
Re-evaluation NGAL & re-evaluation Cystatin C	0.536	0.039 sig

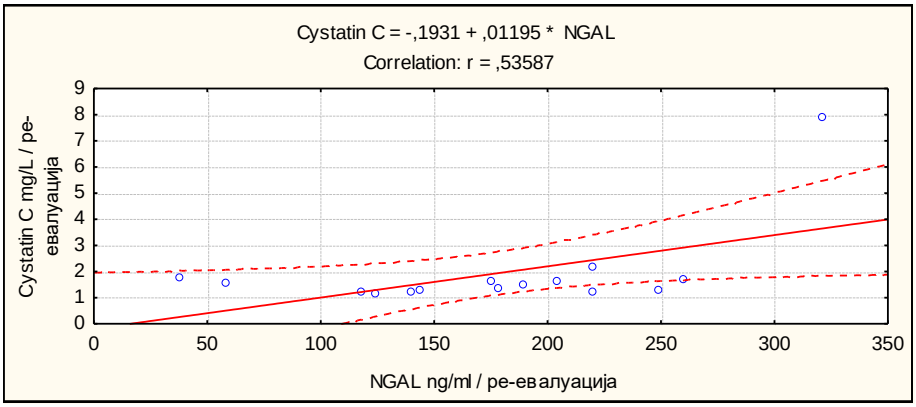

Fig. 7. Correlation of NGAL with Cystatin C - after 6 months

Discussion and conclusion

Microalbuminuria is generally considered to be the earliest marker of the development of diabetic nephropathy and is often associated with significant glomerular damage. However, several studies and lines of evidence suggest that early structural damage to both glomerular and tubular structures may be present in normal albuminuria patients.

The first results of this long-term observational study aimed at assessing the predictability of early markers of renal injury, such as NGAL and CysC, in patients with T2D by detecting renal structural damage long before renal dysfunction occurs.

The study showed that baseline creatinine, microalbuminuria, NGAL, and Cystatin C serum levels were higher in patients diagnosed with DM type 2 and diabetic nephropathy compared to those with and without diabetes and healthy subjects.

Patients in both groups with diabetes (both diabetic nephropathy and non-diabetic nephropathy) were compared in terms of the values of all four markers (creatinine, microalbuminuria, NGAL and Cystatin C) after 6 months of follow-up, and all parameters were higher in the group with diagnosed diabetic nephropathy, with confirmed statistical significance.

In the group with diabetes and diabetic nephropathy, during the re-evaluation after 6 months of monitoring we registered a non-significant increase in the biomarker NGAL (p=0.16), and a significant increase in the biomarker Cystatin C (p=0.016).

The results of the study showed a statistically significant correlation between baseline creatinine values and baseline and control values of Cystatin C (p<0.0001), and creatinine and NGAL values after 6-month re-evaluation (p=0.014), and all these correlations were positive, i.e. with the increase of serum creatinine values the biomarkers NGAL and Cystatin C increased, and vice versa.

The correlation between the two biomarkers NGAL and Cystatin C was statistically insignificant on the first measurement (p=0.16), and significant and direct, i.e. positive on the second measurement, after 6 months (r =0.536, p=0.039). The two markers changed in direct proportion to the serum, with increasing one marker in the serum, the other biomarker increased, and vice versa.
The fact that a certain percentage of normal albuminous patients had elevated values of the biomarkers NGAL and Cystatin C, while a small percentage of microalbuminuria patients had elevated values of NGAL and Cystatin C within normal limits, may reflect different sites of renal impairment during DN.
The use of new biomarkers (NGAL and Cystatin C) as additional tests on existing (creatinine and microalbuminuria) for early diagnosis of DN, speed up effective approaches to management and treatment that are desperately needed to minimize rates of severe cardiorenal morbidity and mortality in patients with T2D. Therefore, these data need to be confirmed by further large-scale longitudinal studies before being integrated into the DN risk assessment in patients with T2D.

Conflict of interest statement. None declared.

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