PROSPERITAS VESTRA FINIS NOSTRA!

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PREDICTIVE ROLE OF NGAL AS AN EARLY MARKER OF RENAL IMPAIRMENT IN PATIENTS WITH TYPE I AND TYPE II DM

ABSTRACT

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The dissertation contains a total of 150 pages, illustrated by 36 figures and 47 tables. The bibliography contains 221 reference sources, 10 of them in Cyrillic and 211 in Latin alphabet.

The research and the laboratory analysis were carried out in the Clinical Medical Diagnostics Laboratory at University General Hospital (UGH) "Sveta Marina" – Varna city

The doctoral candidate works at the Clinical Medical Diagnostics Laboratory at University General Hospital (UGH) "Sveta Marina" and holds the position of "assistant" at the Department of Clinical Laboratory at the Medical University "Prof. Dr. P. Stoyanov" – Varna city. The dissertation was discussed, accepted and referred for defense before a scientific panel by the Departmental Council No. 9, Department of Clinical Laboratory at the Medical University "Prof. Dr. P. Stoyanov" – Varna city, on 18 February, 2022.

The defence of the dissertation will take place on 13 May, 2022.

The materials on the defence are available in the library of the Medical University “Prof. Dr. P. Stoyanov” – Varna and in electronic format on the website of MU – Varna.

Note: In the abstract the numbers of the tables and figures do not correspond of the numbers in the dissertation.

CONTENTS:

ABBREVIATIONS USED

ACR – uAlbumin/uCreatinine ratio

ADA – American Diabetes Association

AER – Albumin excretion rate

BMI – Body mass index

CKD – Chronic kidney disease

CKD-EPI – Chronic Kidney Disease Epidemiology Collaboration

CV – Coefficient of variation

CVD – Cardiovascular Disease

DKD – Diabetic kidney disease

DM – Diabetes mellitus

DN – Diabetic nephropathy

ESRD – End-Stage Renal Disease

GFR – Glomerular filtration rate

ID-MS – Isotopic dilution mass spectrometry

IQR – Interquartile Range

KIM-1 – Kidney Injury Molecule-1

LCN-2 – Lipocalin-2

LLOD – Lower limit of detection

LLOQ – Lower limit of quantification

MDRD – Modification of Diet in Renal Disease

NGAL – Neutrophil gelatinase-associated lipocalin

PETIA – Particle-Enhanced Turbidimetric Immunoassays

SD – Standard deviation

ULOQ – Upper limit of quantitation

UNC – uNGAL/uCreatinine ratio

UTI – Urinary tract infection

WHO – World Health Organization

I. INTRODUCTION

Diabetes mellitus (DM) is a socially significant disease with an increasing incidence that reaches epidemic proportions. One of the main and serious complications of the DM is diabetic kidney disease (DKD), which is associated with increased cardiovascular morbidity (CVM) and the risk of developing end-stage renal disease (ESRD). Early detection and better management of DKD may delay its progression to ESRD and prevent cardiovascular complications. At present, the glomerular filtration rate and the rate of albumin excretion or albumin/creatinine ratio are the main criteria for diagnosis, classification and stratification of the risk of DKD. These indicators demonstrate a number of limitations, necessitating the search and validation of new markers in the diagnosis of DKD.

Diabetic nephropathy is a disease that predominantly affects the glomerulus, but a number of studies indicate the predictive role of tubulo-interstitial lesions in the development and progression of DKD. Neutrophil gelatinase-associated lipocalin (NGAL) is one of the most promising tubular biomarkers in the diagnosis of kidney disease. NGAL is an indicator of renal structural damage, increased plasma and urine NGAL values indicate the degree of subclinical tubular damage and are earlier markers of renal dysfunction compared to classic glomerular indicators. Data in the literature indicate NGAL as a marker with a good diagnostic profile in the diagnosis of DKD. NGAL values in patients with DM correlate with the progression of albumin excretion, with the decline in glomerular filtration rate, with the severity of renal impairment and with the risk of progression to ESRD. NGAL is defined as an early marker of DKD, which detects the development of renal dysfunction prior to increased albumin excretion. However, a number of causes outside the DKD may induce increased expression of NGAL such as obesity, insulin resistance and DM, CVD and urinary tract infections. All this shows the need for further research to clarify the predictive value of NGAL in the diagnosis of DKD, it inspires our interest and forms the basis for the present research.

For clinical practice, the immunoturbidimetric test for NGAL determination is appropriate. In order to introduce the method into routine practice, it is necessary to verify and build methodological and age-dependent reference limits for the Bulgarian population. In order to establish the clinical relevance of NGAL as a marker for renal impairment in DM, it is necessary to assess its diagnostic reliability and the effectiveness of methodically derived cut-off values in distinguishing patients with DKD.

II. PURPOSE AND GOALS

Purpose:

To determine the diagnostic reliability of NGAL as a marker for DKD in patients with DB I and DB II. In this context, we have set some specific goals.

Goals:

1. To introduce and verify immunoturbidimetric method for determination of NGAL of biochemical analyzer ADVIA 1800.
2. To determine age and sex-dependent reference limits for the Bulgarian population of NGAL in plasma and urine as measured by immunoturbidimetric analysis.
3. To evaluate the biological variation of pNGAL, uNGAL and UNC in the reference groups.
4. To determine the diagnostic reliability and clinical applicability of pNGAL, uNGAL and UNC as markers for diagnosis of DKD in patients with DM II.
5. To determine the diagnostic reliability and clinical applicability of pNGAL, uNGAL and UNC as markers for the progression of DKD in patients with DM II.
6. To determine the diagnostic reliability and clinical applicability of pNGAL, uNGAL and UNC as markers for diagnosis of DKD in patients with DM I.
7. To determine the diagnostic reliability and clinical applicability of pNGAL, uNGAL and UNC as markers for the progression of DKD in patients with DM I.

III. MATERIALS AND METHODS

Materials:

This prospective study was conducted at the University General Hospital "Sveta Marina" – Varna city for the period 2019 – 2021 The research protocol has been approved by the Research Ethics Committee at the Medical University – Varna. All study participants received and filled out an informed consent protocol.

For the introduction and verification of latex enhanced immunoturbidimetric analysis, a reagent kit, calibrators and controls of BioPorto Diagnostics A/S (Denmark) were used. The analysis was performed on a biochemical analyzer ADVIA 1800. The calibration kit of the manufacturer Bioporto was used to build a 6-point calibration curve. Two levels of control material were used to verify the test and to validate the analytical series, in diagnostically relevant areas of the control kit of the manufacturer Bioporto.

For the derivation of NGAL reference limits in plasma and urine for the Bulgarian population, a scientific study was performed with:

1. Object of the scientific research – 127 healthy volunteers.
2. Subject of the research – determination of reference limits and assessment of biological variation of NGAL in plasma and urine in the Bulgarian population.
3. Place of scientific research – all volunteers have passed through the Clinical Medical Diagnostics Laboratory at University General Hospital (UGH) "Sveta Marina" – Varna city. Laboratory tests were carried out in the Clinical Medical Diagnostics Laboratory at University General Hospital (UGH) "Sveta Marina" – Varna city.
4. Nature of observation – prospective study.
5. Design of the scientific research – 135 volunteers are involved. The health status of subjects in the reference group was assessed using a questionnaire method and a set of laboratory tests – blood count, glucose, urea, creatinine, total cholesterol, triglycerides, semi-quantitative examination and sediment urine, eGFR was calculated. Blood count and urinalysis results were used as exclusion criteria while all other laboratory parameters were included in the data processing.

   The following criteria are applied:

   5.1. Including criteria: clinically healthy subjects without anamnesis and laboratory evidence of acute or chronic disease and/or intake of nephrotoxic drugs.

   5.2. Excluding criteria: history of acute or chronic kidney disease or other systemic disease with a potential risk of secondary renal injury, malignancies, systemic or local infections, arterial hypertension, thyroid disease, rheumatological diseases, administration of drugs with known nephrotoxic activity or corticosteroid preparations, pregnancy, increased urea, creatinine, glucose, abnormal blood count, abnormal urinary finding, eGFR <60 ml/min/1.73 m2. Criteria for exclusion from the study were found in 5 of the adult volunteers and 3 of the children.

   5.3. The persons who meet the criteria are 127 and are divided into groups as follows:

   5.3.1. Reference group of healthy subjects >18 years old - 85 healthy adults.

   5.3.2. Reference group of healthy subjects < 18 years old - 42 healthy children.

To assess the diagnostic reliability of pNGAL, uNGAL and UNC as markers for DKD in patients with DM, scientific research was carried out with:

1. Object of the scientific research – 167 patients with DM.
2. Subject of the scientific research – assessment of the diagnostic reliability of NGAL as a marker for DKD in patients with DM II and DM I.

3. Place of research – patients are referred by clinicians from the Clinic of Endocrinology and Metabolic Diseases and from the First Children's Clinic of the General University Hospital "Sveta Marina", Varna city. Laboratory tests were carried out in the Clinical Medical Diagnostics Laboratory at University General Hospital (UGH) "Sveta Marina", Varna city.
4. Nature of observation – prospective comparative study.
5. Design of the scientific research – the study included 177 patients with DM II and DM I. In order to assess the health status and for the purpose of the clinical study, patients with DM were tested – blood count, creatinine, urea, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, HbA1C, semi-quantitative examination and sediment of urine, creatinine and albumin in urine, ACR, eGFR and BMI were also calculated. Blood count and urinalysis results were used as exclusion criteria while all other laboratory parameters were included in the data processing.

   The following criteria are applied:

5.1. Inclusion criteria – patients with a confirmed diagnosis of type I DM with a duration of more than 5 years and patients with a diagnosis of type II DM, as per the criteria of ADA and WHO.

5.2. Exclusion criteria: acute and chronic kidney disease outside the DKD, malignancies, systemic or local infections, rheumatological diseases, thyroid diseases, pregnancy, intake of nephrotoxic drugs or corticosteroids, leukocytosis, presence of leukocyturia, eGFR <15 ml/min/1.73m^2. In 7 of the patients with DM II and in 3 of the patients with DM I, exclusion criteria were established.

5.3. Patients meeting the inclusion criteria were 167 and were divided into 2 groups:

5.3.1. Patient group of persons with DM II – 92 adult patients with DM II;

5.3.2. Patient group of persons with DM I – 75 children with DM I.

5.4. Classification of patient groups – patients with DM I and DM II are divided according to the values of ACR, eGFR, HbA1c and the presence of DKD. The mean values of pNGAL, uNGAL and UNC were compared both between individual patient subgroups and with their respective control groups.

5.5. The correlation between pNGAL, uNGAL and UNC with markers for renal impairment (ACR, eGFR, urea, creatinine), for metabolic control (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and for glycaemic control (HbA1c) was evaluated.

5.6. Cut-off values for renal impairment in DM were derived.

5.7. The information received from the participants in the study will be stored in the Department of Clinical Laboratory – Medical University Varna for a period of 5 years.

For the determination of NGAL and the laboratory parameters described, blood was taken under standardised conditions – after a 12h food break, as well as first morning urine. The following biological materials have been used:

1. Complete blood count – obtained in a vacutainer with anticoagulant K_2EDTA from which blood counts and HbA1c were tested.
2. Heparin plasma – obtained in a vacutainer with anticoagulant lithium heparin, which is centrifuged for 15 minutes at 2500G. In heparin plasma, pNGAL, urea, creatinine, total cholesterol, HDL- and LDL-cholesterol and triglycerides were tested.
3. First morning urine, amount: 20 ml. Semi-quantitative chemical testing was performed and sediment in urine was evaluated. Quantitative parameters in urine were determined after centrifugation of the urine for 15 min. at 2500G; concentration of uNGAL, creatinine and albumin were tested in the supernatant.
4. The samples were tested within two hours of receipt of the biological material for all parameters except NGAL. The samples for NGAL – plasma and urine – were transferred to labeled vacutainers without additive. The materials are stored at -20◦C until analysis, for a period not exceeding 3 months. The analysis of pNGAL and uNGAL was performed in stages after collecting a suitable series.

Methods:

To verify the latex enhanced immunoturbidimetric analysis method for the determination of NGAL with ADVIA 1800 biochemical analyzer, the following analytical reliability criteria were evaluated:

1. A calibration curve check and an analytical measurement range check were performed – LLOD (Limit of detection), LLOQ (Lower Limit of Quantification) and ULOQ (Upper Limit of Quantification).
2. Irreproducibility in a series has been assessed.
3. Irreproducibility over time has been assessed.
4. The proportional system error was estimated using the analytical detection method and %recovery was calculated.
5. The transfer of biological material from a high concentration sample to a low concentration sample is determined by % Carry over.
6. Uncertainty and inaccuracy were assessed.
7. Results archiving: All results obtained in the course of the trial – patient results, control results and control cards are systematized, processed and stored in the analyzer software program, in tabular form of EXCEL (Windows Office) files and in paper form.

The following methods have been used to derive reference limits and to determine the diagnostic reliability of the method:

1. Survey method: Prior to receipt of biological material, all study participants were surveyed by the investigator or by clinicians (specialists in endocrinology and pediatric endocrinology), which aims to establish the existence of inclusion criteria in the study.
2. Laboratory analysis – the cited laboratory tests were performed in the Clinical Laboratory at the University General Hospital (UGH) "Sveta Marina", Varna city:

2.1. NGAL in plasma and urine was assayed by latex enhanced immunoturbodimetric analysis on a biochemical analyzer ADVIA 1800.

2.2. UNC is calculated using the formula uNGAL/uCreatinine x 1000, the results are given µg/mmol.

2.3. Urine albumin concentration was assessed by immunoturbodimetric analysis via a biochemical analyzer – ADVIA 1800.

2.4. ACR is calculated using the formula AER/uCreatinine x 1000, the results are given in g/mol.

2.5. Creatinine in plasma and urine was tested by Jaffe – a kinetic method of ADVIA 1800 analyzer, traceable to ID-MS.

2.6. Urea in plasma was determined by a two-stage enzyme urease/UV spectrophotometric test on an ADVIA 1800 biochemical analyzer.

2.7. Total cholesterol was measured by a three-step enzyme reaction with a Trinder in an ADVIA 1800 biochemical analyzer.

2.8. Triglycerides were determined by a three-step enzyme reaction with a Trinder in an ADVIA 1800 biochemical analyzer.

2.9. HDL-cholesterol was determined by direct enzymatic method with elimination/catalase in biochemical analyzer ADVIA 1800.

2.10. LDL-cholesterol was calculated using the Friedewald's formula for triglycerides < 4.5 mmol/l – LDL-chol=TChol–(HDL-chol+TG/2.2). At triglyceride value > 4.5 mmol/l, LDL-cholesterol was measured by direct enzymatic method with elimination/catalase in biochemical analyzer ADVIA 1800.

2.11. HbA1c was determined by a method standardized according to the DCCT/NGSP in the ADVIA 1800 biochemical analyzer. Results are presented in %, calculated from the HbA1c/Total Hemoglobin (THb) ratio. HbA1c was measured via turbidimetric immunoassay. THb was measured by a colorimetric method.

2.12. Blood count – blood counts were determined on a 5-diff hematology analyzer – Sysmex XN 1000, using the principles of fluorescence flow cytometry using a semiconductor laser and hydrodynamic focusing.

2.13. Semi-quantitative chemical testing of urine and sediment in urine was determined on a combined H-800/FUS-100 system operating on the principle of refractometry with 11 parameter test strips for dry chemistry and flow cytometry.

2.14. For the calculation of eGFR in adults, the formula CKD-EPI was used. eGFR = 141 min(Scr/κ, 1)α×max(Scr/κ, 1)-1.209×0.993Age×1.018 (if female), in which Scr – serum creatinine in μmol/l, κ – 61.9 for females and 79.6 for males, α – -0.329 for females and -0.411 for males, min –the minimum of Scr/κ or 1, max –the maximum of Scr/κ or 1.

2.15. The updated formula of 'Bedside Schwartz' and the formula CKD-EPI40 were used to calculate eGFR in children.

$eGFR_{(Bedside\ Schwartz)}$ =(36.2×height in cm)/serum creatinine in μmol/l

$eGFR_{(CKD\text{-}EPI40)}$ is based on the CKD-EPI equation, but with age-adjusted creatinine at about the age of 40, the creatinine correction was performed using the following formulas:

in boys: $Ln(creatinine)=Ln(creatinine)+0.259\times(40\text{-}age)-0.543\times Ln(40/age)-0.00763(40^2-age^2)+0.0000790\times(40^3-age^3)$.

in girls: $Ln(creatinine)=Ln(creatinine)+0.177\times(40\text{-}age)-0.223\times Ln(40/age)-0.00596(40^2-age^2)+0.0000686\times(40^3-age^3)$.

3. Statistical methods – in statistical data processing, the capabilities of the statistical package SPSS 27 are used and the following methods are applied.

3.1. Shapiro-Wilk's test to evaluate the normality of the distribution of continuous variables.

3.2. Continuous variables that follow normal distribution are represented by mean and standard deviation. The variables that do not follow normal distribution are represented by median and interquartile range (IQR).

3.3. The mean values were compared using Student-Fisher's t-test to establish statistically significant differences in categorical variables.

3.4. Non-parametric tests of Mann-Whitney and Kruskal-Wallis were used to compare two or more groups of variables, respectively, that did not follow a normal distribution.

3.5. To determine the strength and direction of dependence, correlation analysis was applied – Pearson's correlation coefficient for variables with normal distribution and Spearman Rho's rank correlation coefficient for variables that do not follow normal distribution.

3.6. A linear regression analysis for interval dependent variable and logistic regression and a discriminant analysis for category dependent variable were used to determine the degree of dependence and predict the values of a dependent variable.

3.7. Receiver-Operating Characteristic (ROC) curves and Area Under the Curve (AUC) were used to evaluate the sensitivity and specificity of laboratory parameters.

3.8. A critical significance level of $\alpha=0.05$ or $p<0.05$ was used.

IV. RESULTS

1. Verification of the immunoturbidimetric method for the determination of NGAL

The detectable minimum (LLOD) of NGAL is determined by taking into account the standard deviation (SD) of 10 blanks measured in parallel. LLOD is calculated by the formula: LLOD = 3.3*SD-the blank sample, and is equal to 6.00 ng/ml. The Lower Limit of Quantification (LLOQ) is defined as a signal exceeding five to ten times the background noise and is in the range of 12.00 ng/ml to 18.00 ng/ml.

The resulting LLOQ values (12-18 ng/ml) and the manufacturer's upper limit of quantification (ULOQ - 3000 ng/ml) were evaluated to verify the calibration curve. 5 parallel measurements were made on three samples with target values of 12 ng/ml, 17.3 ng/ml and 3000 ng/ml. The LLOQ results for the two samples used are: $\bar{x}$ = 10.46 ng/ml, CV = 17.52 % ($_{x0}$ =12 ng/ml) and $\bar{x}$ = 16.42 ng/ml, CV = 10.12 % ($_{x0}$ =17.3 ng/ml). The results for ULOQ are: $\bar{x}$ = 2926.48 ng/ml and CV = 1.55% ($_{x0}$ =3000 ng/ml). The results obtained were in accordance with international requirements (CV up to 20% for LLOQ and up to 15% for ULOQ) and a linearity of the test from 12 ng/ml to 3000 ng/ml was applied in this study.

For the assessment of irreproducibility in a series, 10 parallel measurements were carried out in a series of 4 selected samples. For analysis, 1 urine sample and 3 plasma samples were used in a reference area, clinically significant area and high area, respectively. The samples were analysed under identical conditions (date, reagent lot No, operator). The following was calculated: mean value ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV%) (Table 1).

Table 1. Statistical data from irreproducibility in a series

Sample/concentration area	Number of measurements	$\bar{x}$	SD	CV%
urine/low	10	41.11	2.65	6.43
plasma/low	10	31.37	1.65	5.26
plasma/clinically significant	10	94.26	1.74	1.85
plasma/high	10	1221.75	22.27	1.82

The control materials of the manufacturer Bioporto, in two concentration areas, were used to assess the irreproducibility in time. The control materials were analysed in 21 working days under identical conditions prior to the patient series. The following was calculated: mean value ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV%) (Table 2).

Table 2. Data from the statistical processing of reproducibility in time

Sample/concentration area	Number of measurements	$\bar{x}$	SD	CV%
low control	21	202.69	9.04	4.5
high control	21	507.03	13.46	2.7

Control materials of the manufacturer Bioporto were used to assess unreliability and inaccuracy in two diagnostically significant areas, analysed in 21 working days. A percentage deviation (d%) of the mean value ($\bar{x}$) from the manufacturer's declared value (x_0) is calculated (Table 3). Intra-laboratory quality control was carried out in accordance with the Westgard criteria with two levels of control material.

Table 3. Assessment of unreliability with control material NGAL Test^{TM} Control

Control material	Number of measurements	x_0	$\bar{x}$	d%
low control	21	202	202.69	0.34
high control	21	505	507.03	0.40

The analytical detectability method was used to estimate the proportional system error by calculating %recovery. 4 samples were prepared by mixing patient samples and concentrated solutions. The test was performed with both types of biological material – plasma and urine (Table 4).

Table 4. Assessing analytical detectability

Sample type ng/ml	Additive ng/ml	Sample-to-Additive Ratio	Added concentration	Concentration found	Analytical yield %
plasma - 31	1222	0.95:0.05	59.5	56.2	95
plasma - 31	1222	0.90:0.10	119	120.5	101
urine - 41	520	0.90:0.10	48	47.1	98

%Carryover was selected to assess the transfer of biological material from samples with high values to samples with low values of the tested benchmark. The following approach was used: sample A with high concentration was analysed 3 times in succession (A1, A2, A3), followed by a sample with low values, also analysed 3 times in succession (B1, B2, B3). %Carryover is estimated by the formula %Carryover = B1-B3/A3-B3x100 (Table 5).

Table 5. %Carryover assessment

	1	2	3
Sample A	1208	1166	1236
Sample B	28.5	32.3	32.1
% Carryover	-0.29 %		

2. NGAL - reference interval in adults. Biological variation

90 adult volunteers were included in the reference group, 5 had significant leukocyturia and were excluded from subsequent data processing. The cohort meeting the inclusion criteria in this study included 85 subjects aged 34-75 years. (52.9±8.3), of which 44 females and 41 males. A characteristic of the adult control group is presented in Table 6 (Table 6).

Table 6. Characteristics of the adult control group

Indicator	Units of Measure	Mean ±SD / median(IQR)
number (females:males %)	N (%)	85 (52%: 48%)
years	years	52.96 ± 8.39
glucose	mmol/l	5.25 ±0.65
urea	mmol/l	4.96 ±1.21
creatinine	µmol/l	73.5 ±16.66
eGFR	ml/min/1.73m²	91.10±14.78
total cholesterol	mmol/l	5.37±1.00
triglycerides	mmol/l	1.46±1.24
pNGAL	ng/ml	72.03±24.02 / 72.65(33.38)
uNGAL	ng/ml	21.19±15.74 / 14.50(11.55)
UNC	µg/mmol	2.08±1.55 / 1.75(1.25)

The statistical check for normal distribution of results for the indicators pNGAL, uNGAL and UNC, as well as the visual assessment of the histograms and P-Plots, showed the lack of Gaussian distribution. The Shapiro-Wilk's test ($p<0.05$) demonstrated coefficients for asymmetry and excess with z-value >1.96, which rejected the null hypothesis of normal distribution for all three indicators. Subsequent processing was performed after data transformation and non-parametric analysis methods. After statistical processing of the results and normalization of the distribution, a percentile method for the calculation of the reference limits was applied (Fig. 1). The derived reference values correspond to 2.5 and 97.5 percentiles, with a 95% confidence interval. The reference limits for the entire reference group examined were: for pNGAL – 25-119.49 ng/ml, for uNGAL< 52.37 ng/ml, UNC <5.16 µg/mmol.

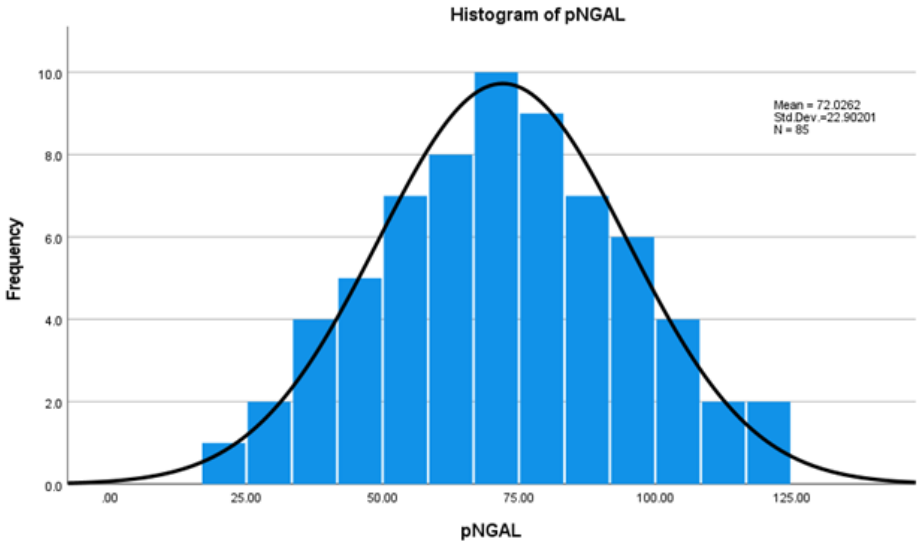


Fig. 1. A histogram representing the normal distribution of transformed pNGAL results in the reference group >18 years.

The median and interquartile range of pNGAL, uNGAL and UNC across the reference group are as follows: 72.65 (52.38-85.75) ng/ml, 14.50 (12.00-23.55) ng/ml and 1.75 (1.25-2.33) µg/mmol. The median pNGAL in females was 73.20 (51.30-85.30) ng/ml and in males it was 72.50 (52.25-89.20) ng/ml. The median uNGAL in women was 19.50 (12.30-24.10) ng/ml and in men it was 12.55 (12.00-23.20) ng/ml. The median UNC in women was 1.96 (1.56-2.81) µg/mmol and 1.22 (0.90-1.97) µg/mmol in men (Fig.2). Mann-Whitney test results showed statistically significantly higher UNC scores in females compared to males U= 207.0 p = 0.003 r = -0.402. The gender-differentiated UNCs were: in females <6.34 µg/mmol and in males < 3.36 µg/mmol.

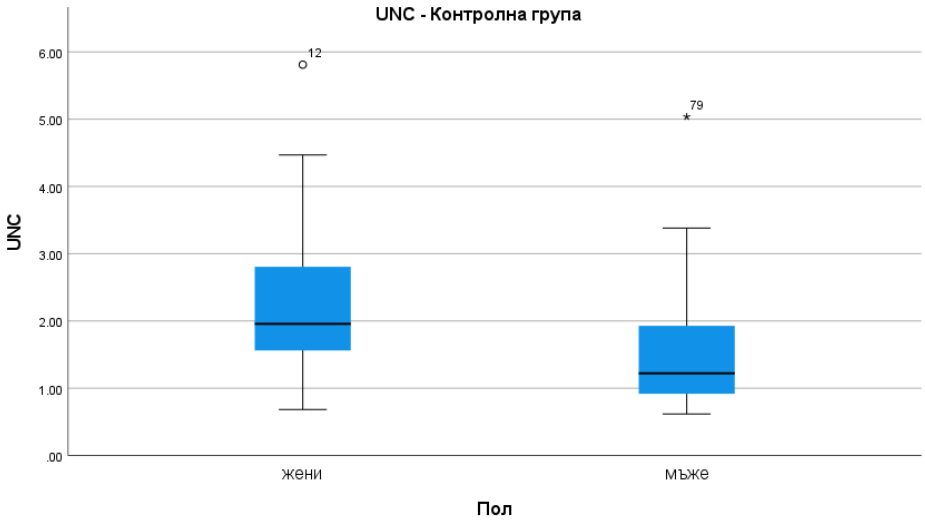


Fig. 2. Distribution of UNC scores for women and men in the reference group

The median uCreatinine in females was 9695 (6073-14241) µmol/l and in males it was 13718 (10644-17132) µmol/l (Fig. 3). Mann-Whitney test results showed statistically significantly higher uCreatinine scores in males compared to females U= 252.0, p = 0.023, r = -0.303.

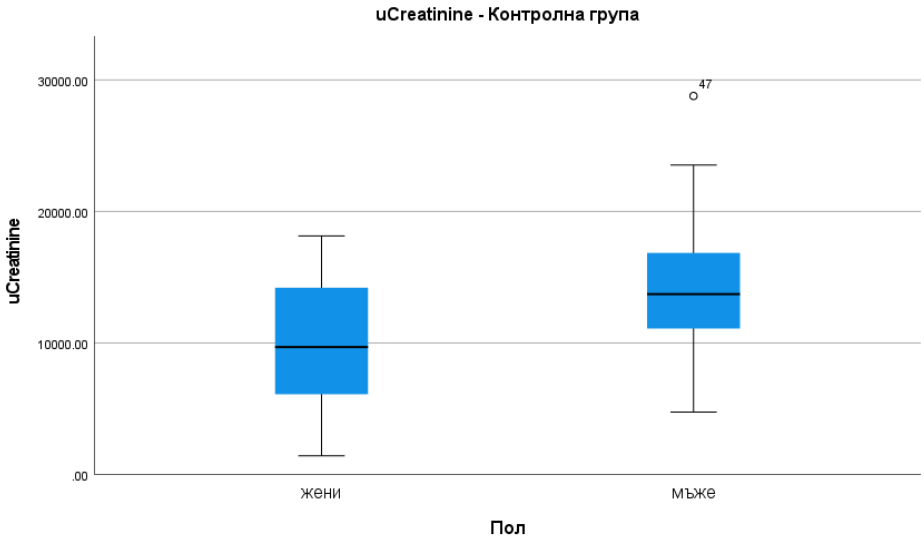


Fig. 3. Distribution of uCreatinine scores for women and men in the reference group

Individuals are divided into three age groups: ≤ 49 years (N=30), 50-59 years (N=36) and ≥60 years (N=19). Depending on age, the median and interquartile range of pNGAL, uNGAL and UNC are as follows: in subjects ≤ 49-pNGAL is 72.4 (49.47-83.42) ng/ml, uNGAL is 18.15 (12.00-24.70) ng/ml and UNC is 1.45 (1.00-2.16) µg/mmol, in the age decade 50-59 – pNGAL is 63.00 (50.60-74.10) ng/ml, uNGAL is 13.15 (12.00-17.42) ng/ml and UNC is 1.77 (0.93-2.60) µg/mmol and in subjects ≥ 60 – pNGAL is 87.90 (74.20-97.30) ng/ml, uNGAL is 23.75 (12.00-33.17) ng/ml and UNC is 1.91 (1.74-2.48) µg/mmol (Fig. 4). The median pNGAL in all subjects below 60 years of age is 67.70 (50.60-81.60) ng/ml and in subjects above 60 years of age is 87.90 (74.20-97.30) ng/ml. Mann-Whitney test results showed significantly higher pNGAL scores in subjects over 60 years of age compared to those under 60 years of age U=200.0 p<0.001 r = -0.40. In order to investigate the association between pNGAL values with age and eGFR, the Spearman Rho rank correlation coefficient was used. The correlation analysis showed a statistically significant negative correlation between age and eGFR (rho = -0.473 p <0.001), between pNGAL and eGFR (rho= - 0.354 p =0.003), as well as a positive correlation between pNGAL and age (rho=0.255 p =0.035). Differentiation of the control cohort into three groups led to a reduction in the number of subjects in each group and limited the possibility of setting age dependent reference limits for pNGAL.

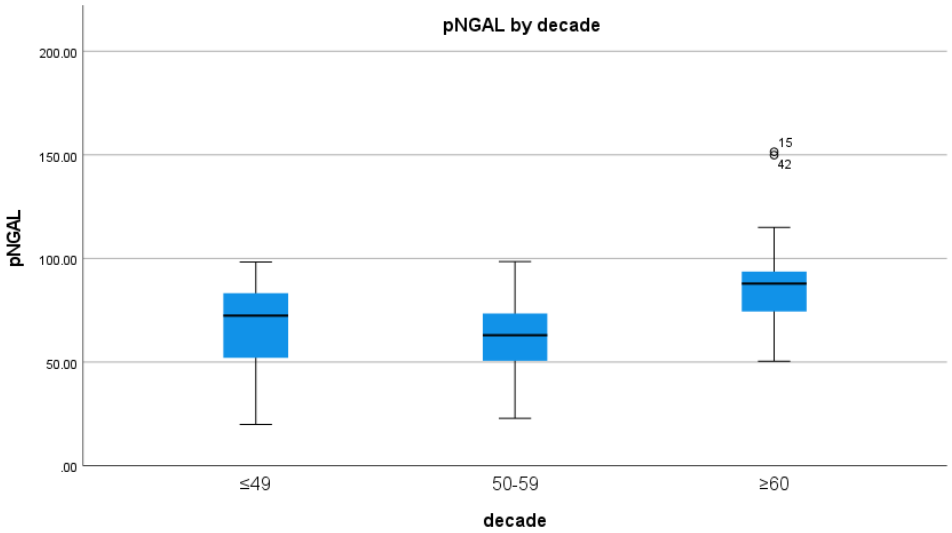


Fig. 4. Distribution of pNGAL results by age groups

Five subjects in the study had significant leukocyturia and were excluded from data processing. The mean value of uNGAL measured in these patients was $\bar{x}$=141.7 ng/ml and UNC – $\bar{x}$ =23.32 µg/mmol. The results obtained for uNGAL and UNC are significantly higher than the defined reference limits (Fig. 5).

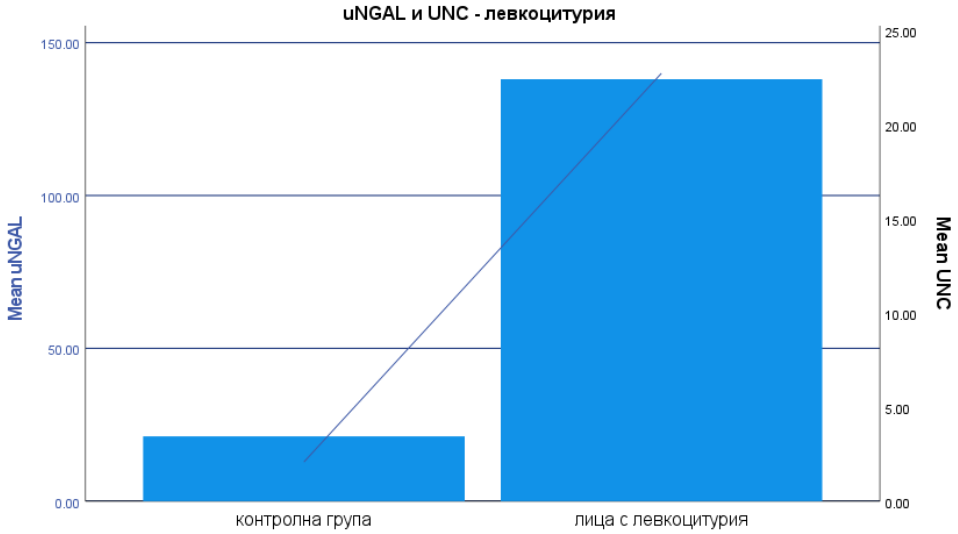


Fig. 5. Mean - uNGAL and UNC in subjects with and without leukocyturia

3. NGAL - reference interval in children. Biological variation

45 children were included in the reference group, 3 of them were found to have leukocytosis, significant leukocyturia and proteinuria and were excluded from further data processing. The cohort meeting the inclusion criteria in this study comprised of 42 individuals aged 5 to 17 (12.50±3.69), of whom 21 girls and 21 boys (Table 7).

Table 7. Characteristic of the control group – children

Indicator	Units of Measure	Mean ±SD /Median(IQR)
number (girls:boys)	N (%)	42 (50%:50%)
years	years	12.50±3.69
urea	mmol/l	3.94 ±1.05
creatinine	µmol/l	59.66 ±16.22
eGFR	ml/min/1.73m²	94.11±16.42
glucose	mmol/l	4.69 ±0.71
total cholesterol	mmol/l	4.28±0.96
triglycerides	mmol/l	0.95 ±0.46
pNGAL	ng/ml	62.55±20.16/ 66.15(28.45)
uNGAL	ng/ml	19.96±13.93/ 13.70(10.35)
UNC	µg/mmol	1.72±0.90/ 1.47(1.06)

The statistical check for the normal distribution of results for the indicators pNGAL, uNGAL and UNC, as well as the visual assessment of the histograms and P-Plots showed normal distribution of results for pNGAL and the absence of Gaussian distribution of results for uNGAL and UNC (Fig. 6). The Shapiro-Wilk's test ($p<0.05$) demonstrated coefficients for asymmetry and excess with z-value >1.96, which rejected the null hypothesis of normal distribution of the uNGAL and UNC results. Subsequent processing of the results for uNGAL and UNC was carried out following a two-step approach to transforming and normalizing the data distribution. The derived reference values, corresponding to 2.5 and 97.5 percentiles, at the 95% confidence interval in the control group below 18 years of age were as follows: pNGAL < 96.88 ng/ml, uNGAL < 47.30 ng/ml, UNC < 3.48 µg/mmol.

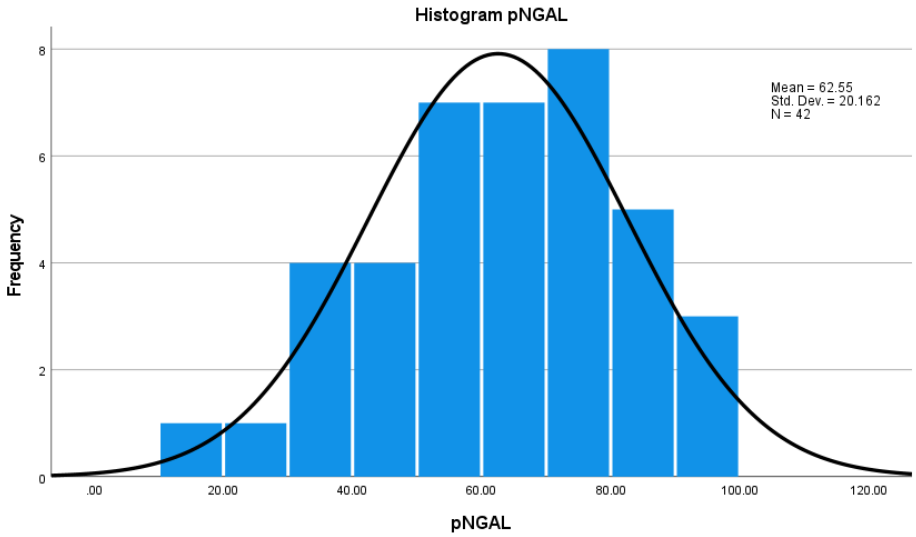


Fig. 6. Histogram representing the normal distribution of pNGAL results in the reference group – less than 18 years.

The median and interquartile range of pNGAL, uNGAL and UNC across the cohort were as follows: 66.15 (48.78-77.23) ng/ml, 13.70 (12.00-22.35) ng/ml and 1.47 (1.15-2.21) µg/mmol. Student-Fisher's T-test found no significant difference between pNGAL scores at gender differentiation (t = -1.49 p= 0.146). The Mann-Whitney test showed no statistically significant difference in uNGAL and UNC values between the subgroups – girls and boys (U=148.5 p=0.094 and U= 198.0p=0.754). There was no significant correlation of all three markers given the age (pNGAL – r =0.140 p=0.390, uNGAL – rho =0.129 p= 0.422 and UNC – rho = -0.162 p= 0.313).

4. Diagnostic reliability of NGAL in the diagnostics of DKD in DM II

99 subjects with MD II were targeted to participate in the study, but 7 patients were found to have exclusion criteria. The individuals with DM II, meeting the inclusion criteria, were 92 patients, aged 18 to 89 (58.28±14.69 years). The gender distribution in the group was: 55% (N=51) middle-aged women 59.73 ±16.98 yrs and 45% (N=41) middle-aged men 56.49±11.17 yrs. (Table 8).

Table 8. Characteristics of the patient group with DM II

Indicator	Patients DM II mean±SD / median (IQR)	Control group >18 yrs. mean±SD / median (IQR)	p
number	92	85	
gender female:male (%)	55%:45%	52%:48%	=0.884
years	58.28±14.69 yrs.	52.96 ± 8.39	<0.004
glucose (mmol/l)	11.98 ±6.49	5.25 ±0.65	<0.001
urea (mmol/l)	7.77 ±4.08	4.96 ±1.21	<0.001
creatinine (µmol/l)	94.82 ±41.01	73.5 ±16.66	<0.001
eGFR (ml/min/1.73m²)	73.04 ±24.68	91.10±14.78	<0.001
total cholesterol (mmol/l)	5.22 ±1.79	5.37±1.00	=0.508
triglycerides (mmol/l)	3.05 ±2.79	1.46±1.24	<0.001
HDL-cholesterol (mmol/l)	1.07 ±0.31	*	*
LDL-cholesterol (mmol/l)	2.79±1.17	*	*
AER (mg/l)	201 ±525	*	*
HbA1c (%)	9.32 ±2.39	*	*
ACR (g/mol)	29.06 ±62.96	*	*
pNGAL (ng/ml)	97.58±63.42/79.10(64.98)	72.03±24.02/72.65(33.38)	p=0.044
uNGAL (ng/ml)	59.07 ±72.04/32.30(67.15)	21.19±15.74/14.50(11.55)	p<0.001
UNC (µg/mmol)	10.62±15.14/4 .78(9.05)	2.08±1.55/1.75(1.25)	p<0.001

The included persons with MD II are divided into subgroups according to – ACR, eGFR and the presence of DKD. Compared to the ACR level, three groups were formed: A1 at ACR <3 g/mol, A2 at ACR – 3-30 g/mol and A3 at ACR >30 g/mol. Compared to eGFR, two groups were formed: ≤G2 at eGFR ≥60 ml/min/1.73m² and ≥G3 at eGFR <60 ml/min/1.73m². Patients were divided into only

two categories depending on eGFR, as their distribution from G1 to G4 (G5 being the exclusion criterion for the study) reduced the number of subjects in the individual subgroups, limiting the possibility of statistical data processing (Fig. 7).

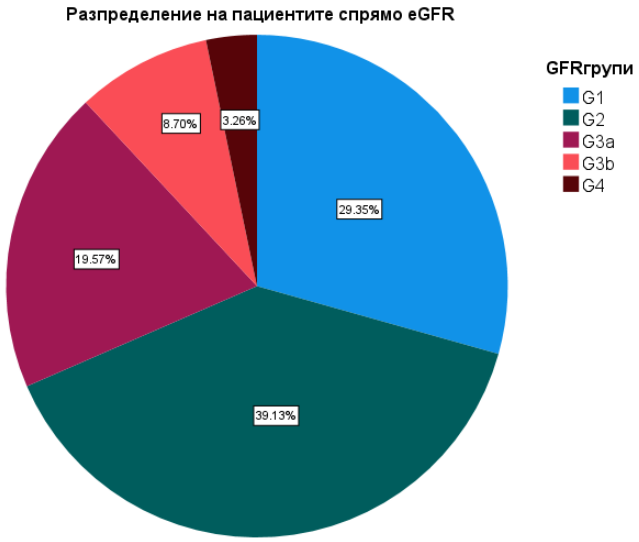


Figure 7. Distribution of patients according to eGFR – category G1 to G4

According to the KDIGO 2020 guidelines, the criteria adopted in this study for the diagnosis of DKD are ACR >3 g/mol and/or eGFR <60 ml/min/1.73m². According to the criteria thus defined, two groups were formed – DM II without DKD and DM II with DKD (Table 9)

Table 9. Characteristics of the groups of patients with DM II

Category	Criteria	Mean±SD	N (women:men)	Age
A1	ACR <3 g/mol	ACR - 1.14±0.54	42 (21:21)	59.12 ±13.31
A2	ACR-3 – 30 g/mol	ACR – 9.88±7.45	28 (20:8)	55.00 ±17.59
A3	ACR >30 g/mol	ACR -110.48±93.00	21 (10:11)	60.42 ±13.17
≤G2	eGFR ≥60 ml/min/1.73m²	eGFR – 87.25±16.71	63 (36:27)	54.57 ±13.14
≥G3	eGFR <60 ml/min/1.73m²	eGFR - 46.41±11.53	29 (15:14)	65.25 ±15.10
DM without DKD	ACR <3 g/mol and eGFR ≥60 ml/min/1.73m²	ACR – 1.13±0.50 eGFR – 89.03±15.50	36 (16:20)	56.58±11.95
DM with DKD	ACR >3 g/mol and/or eGFR <60 ml/min/1.73m²	ACR - 47.34±75.80 eGFR - 62.77±24.10	56 (35:21)	59.38 ±16.22

The statistical check for normal distribution of results for pNGAL, uNGAL and UNC across the cohort of patients with DM II and across subgroups showed the lack of Gaussian distribution. The Shapiro-Wilk's test ($p<0.05$) demonstrated coefficients for asymmetry and excess with z-value >1.96, which rejected the null hypothesis of normal distribution for all three indicators in each of the examined subgroups. The subsequent statistical processing of the data was carried out using non-parametric methods of analysis.

4.1. pNGAL as a marker for the diagnosis of DKD in patients with DM II

The median and interquartile range of pNGAL across the patient cohort was 79.10 (57.30-122.28) ng/ml. Mann-Whitney test results showed statistically significantly higher pNGAL scores in patients with DM II compared to the control group (79.10 vs 72.03 ng/ml) U= 1964.0 p = 0.044 r = -0.171.

The median and interquartile range of pNGAL in the three subgroups separated by ACR are: A1 – 71.10 (52.30-105.30) ng/ml, A2 – 82.10 (51.60-102.47) ng/ml and A3 – 121.70 (66.65-171.20) ng/ml. TheKruskal-Wallis test found no statistically significant difference in pNGAL concentration between subgroups of patients ($\chi 2(^2) = 4.250$, p= 0.119). Only patients in A3 (121.70 vs 72.03 ng/ml) U= 332.0p=0.007 r = -0.293 had significantly higher pNGAL scores compared to the control group.

The median and interquartile range of pNGAL in the two subgroups separated according to eGFR were as follows: ≤G2 – 69.8 (52.60-93.60) ng/ml and ≥G3 – 121.90 (77.70-174.30) ng/ml. The Mann-Whitney test found a statistically significant difference in pNGAL concentration between the subgroups of patients U=278.0 p<0.001 r = -0.406. Compared to the control group, significantly higher values for pNGAL had only the patients in subgroup ≥G3 (121.90 vs 72.03 ng/ml), U=319.0 p<0.001 r = -0.443 (Fig. 8).

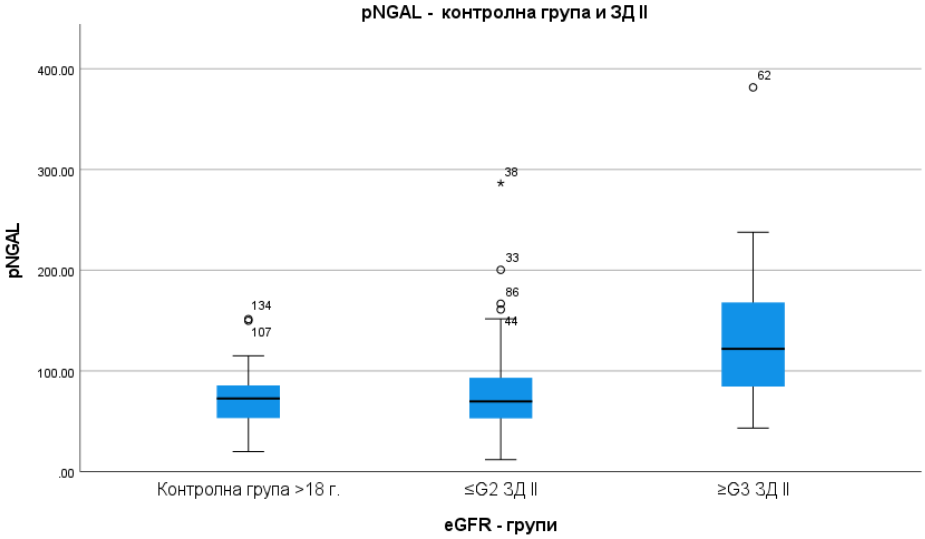


Figure 8. Distribution of pNGAL results in the control group and in patients with DM II, divided according to eGFR

The median and interquartile range of pNGAL in patients with DM II without DKD is 70.45 (53.42-93.80) ng/ml and in patients with DKD it is 93.55 (60.27-138.65) ng/ml. The Kruskal-Wallis test found that the concentration of pNGAL in individual patient subgroups and the control group differed statistically significantly between each other ($\chi 2(^2) = 7.675$p=0.022). After administration of a post-hoc test, this difference proved to be significant only between patients with DKD and the control group (93.55 vs 72.03 ng/ml) U= 918.50 p=0.005 r = -0.270 (Fig. 9)

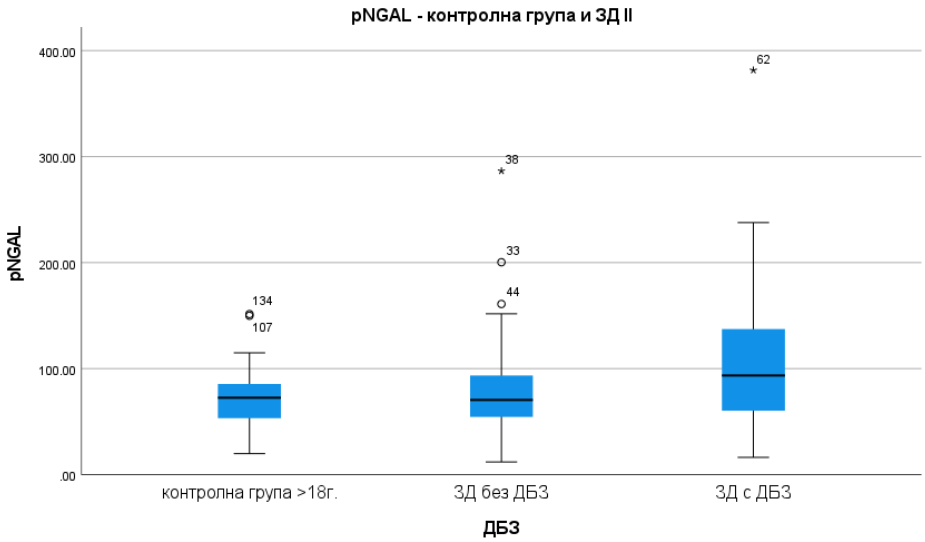


Figure 9. Distribution of pNGAL scores in the control group and in patients with DM II with and without DKD.

The ROC analysis demonstrated that pNGAL had good diagnostic effectiveness in differentiating patients with DM II with decreased eGFR (eGFR<60 ml/min/1.73m²) from those with preserved eGFR (eGFR≥60 ml/min/1.73m²), with AUC-ROC – 0.753. At cut-off- 121.65 ng/ml, a value above the defined upper limit of the reference interval, the test has diagnostic sensitivity and specificity – 57% and 84%, ratio of positive and negative probability – LR+ 3.64 and LR- 0.50 and positive and negative predictive value – 71% and 86% in the distinction of patients with DM II with decreased eGFR. The diagnostic effectiveness with which a pNGAL value >121.65 ng/ml correctly identifies patients with DM II with eGFR <60 ml/min/1.73m2 is 82% (Fig. 10).

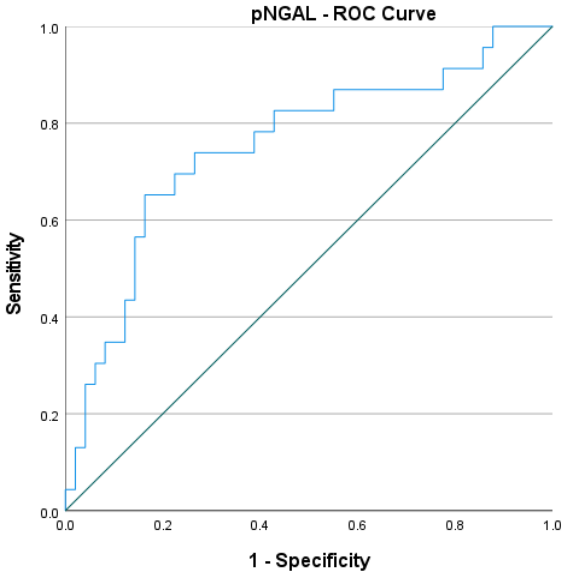


Figure 10. ROC curve of pNGAL in differentiating subjects with DM II with decreased eGFR (eGFR <60 ml/min/1.73m^2)

pNGAL demonstrated poor diagnostic effectiveness in differentiating DM II patients with albuminuria from those with normoalbuminuria (AUC-ROC-0.580). The application of pNGAL alone has sufficient to poor diagnostic effectiveness to detect patients with DKD among patients with DM II with AUC-ROC-0.619. In view of this unsatisfactory reliability of the marker, no cut-off values were derived.

4.2. uNGAL and UNC as markers for the diagnosis of DKD in patients with DM II

The median and interquartile range of uNGAL and UNC across the patient cohort were 32.30 (17.75-84.90) ng/ml and 4.78 (2.26-11.31) µg/mmol. Mann-Whitney test results showed that patients with DM II scored significantly higher for uNGAL and UNC than the control group (32.30 vs 14.50 ng/ml) U= 1178.5 p<0.001 r = -0.428 and (4.78 vs 1.75 µg/mmol) U=759.0 p<0.001 r = -0.575.

The median and interquartile range of uNGAL in the three subgroups of patients separated according to ACR are: A1 – 19.75 (12.45-37.25) ng/ml, A2 – 41.25 (19.02-80.87) ng/ml and A3 – 91.30 (35.80-136.50) ng/ml. The Kruskal-Wallis test found that the concentration of uNGAL in individual patient subgroups and the control group differed statistically significantly between each other ($\chi^2(3) = 47.17$, p<0.001). Following the post-hoc test, this difference proved to be significant for all comparisons between the groups except between A2 and A3 (U= 151.00 p=0.022) and between A1 and the control group (U= 846.00 p=0.039). Patients in subgroup A1 scored significantly higher than in A2 and A3 (19.75 vs 41.25 ng/ml), U= 287.00 p = 0.007 r = -0.335 and (19.75 vs 91.30 ng/ml), U=116.00 p<0.001 r = -0.592. Compared to the control group, significantly higher scores were obtained by subgroups A2 and A3 (41.25 vs 14.50 ng/ml), U= 257.00 p<0.001 r = -0.49 and (91.30 vs 14.50 ng/ml), U=75.00 p<0.001 r = -0.675 (Bonferroni correction for α compared to 4 groups – statistical significance p≤0.008) (Fig. 11).

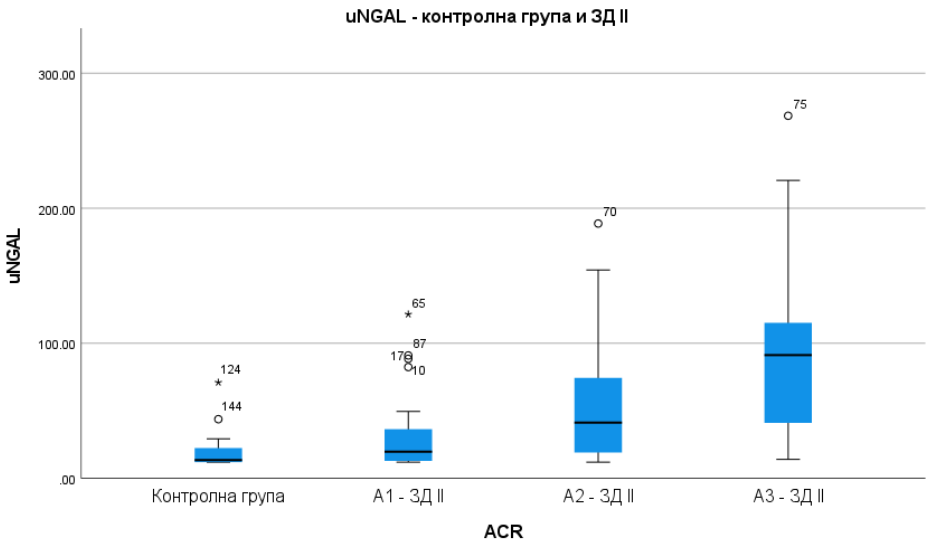


Figure 11. Distribution of uNGAL results in the control group and in patients with DM II, divided according to ACR

The UNC ratio has the following values for median and interquartile range in individual patient subgroups: A1 – 2.52 (1.83-4.32) µg/mmol, A2 – 7.86 (4.11-16.46) µg/mmol and A3 – 10.94 (7.63-33.26) µg/mmol. The Kruskal-Wallis test found a statistically significant difference in UNC concentration between subgroups of patients and the control group ($\chi 2(^3) = 74.59 p<0.001$). Following the post-hoc test, this difference proved to be significant for all comparisons between the groups, except between groups A2 and A3 (U=148.00 p=0.018). Patients in subgroup A1 scored significantly higher than those in A2 and A3 (2.52 vs 7.86 µg/mmol), U= 172.00 p<0.001 r = -0.534 and (2.52 vs 10.94 µg/mmol), U=51.00 p<0.001 r = -0.717. UNC scores in each of the subgroups from A1 to A3 were statistically significantly higher than the control group, including A1 (2.52 vs 1.75 µg/mmol), U= 635.0 p<0.001 r = -0.368 (Bonferroni correction for α compared for 4 groups, statistical significance p≤0.008). Patients with DM II were further separated by gender; the medians of UNC in women in the individual groups were: A1- 3.98 (2.22-5.96) µg/mmol, A2 – 8.28 (4.48-18.11) µg/mmol and A3 – 16.48 (9.13-33.78) µg/mmol, and in men A1- 1.93 (1.73-2.76) µg/mmol, A2 – 4.49 (1.65-11.36) µg/mmol and A3 – 10.30 (4.95-32.86) µg/mmol. In each subgroup, UNC values were higher in females compared to males, but the difference was significant only in subgroup A1 (U=77.00 p<0.001 r = -0.525) and decreased with the progression of albumin excretion (A2-p = 0.119 and A3-p =426). No significant difference in UNC values was found between sex-differentiated subgroups A2 and A3 (Fig. 12).

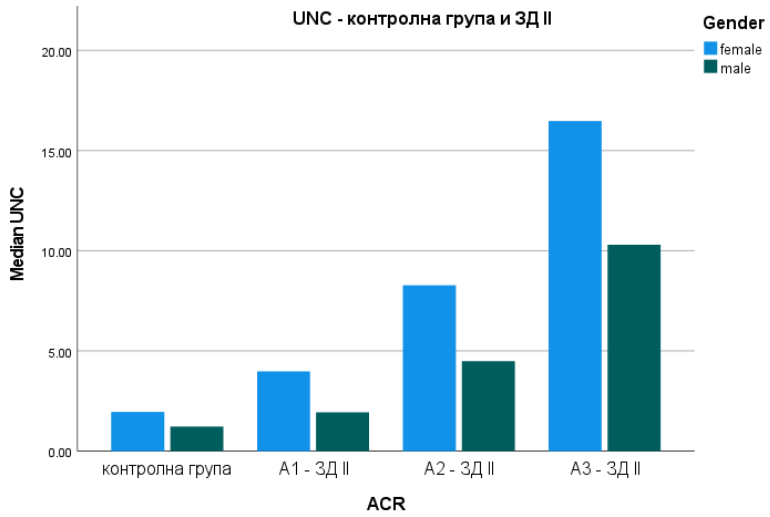


Figure 12. Median UNC in females and males in the control group and in patients with DM II, divided according to ACR.

The median uCreatinine in females was 4670 (3730-9163) µmol/l and in males it was 8444 (5604-11893) µmol/l (Fig. 13). The Mann-Whitney test found that males scored statistically significantly higher than females U= 687.00 p = 0.012 r = -0.264.

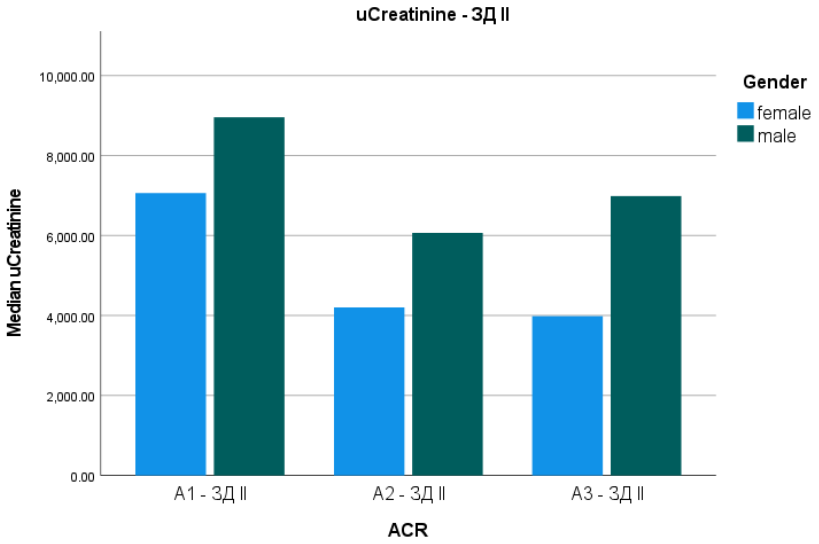


Figure 13. Median uCreatinine in females and males in patients with DM II divided according to ACR.

The median and interquartile range of uNGAL in the two subgroups separated by eGFR were: ≤G2 – 28.45 (16.80-49.72) ng/ml and ≥G3 – 44.20 (22.55-109.15) ng/ml. The Kruskal-Wallis test found a statistically significant difference in uNGAL concentration between the control group and the patient subgroups ($\chi 2(^2)$ = 35.830, p<0.001). Following administration of a post-hoc test, this difference proved to be significant only in relation to the control group. Results for uNGAL in the patient groups ≤G2 and ≥G3 were statistically significantly higher than in the control group (28.45 vs 14.50 ng/ml), U= 877.0 p<0.001 r = -0.383 and (44.20 vs 14.50 ng/ml), U= 301.0 p<0.001 r = -0.519).

The UNC ratio has a median and interquartile range in the subgroup ≤G2 – 4.39 (2.03-12.54) µg/mmol and in the subgroup ≥G3 – 6.21 (3.99-10.46) µg/mmol. The Kruskal-Wallis test found a statistically significant difference in UNC values between the control group and the patient subgroups ($\chi 2(^2)$ = 44.894p<0.001). Following administration of a post-hoc test, this difference proved to be significant only in relation to the control group. UNC scores in the patient groups ≤G2 and ≥G3 were statistically significantly higher than in the control group (4.39 vs 1.75 µg/mmol), U= 573.0 p<0.001 r = -0.546 and (6.21 vs 1.75 µg/mmol), U= 186.0 p<0.001 r = -0.629. The UNC median in females in subgroup ≤G2 is 6.17 (3.43-13.46) µg/mmol and in ≥G3 it is 7.09 (4.35-17.35) µg/mmol, and in males in ≤G2 it is 2.21 (1.80-4.94) µg/mmol, and in ≥G3 it is 4.78 (1.97-10.04) µg/mmol. No significant difference in UNC values was found between sex-differentiated subgroups ≤G2 and ≥G3. UNC values were higher in females compared to males in each of the subgroups, but the difference was significant only in subgroup ≤G2 (U= 215.0 p=0.006 r = -0.366) (Fig. 14).

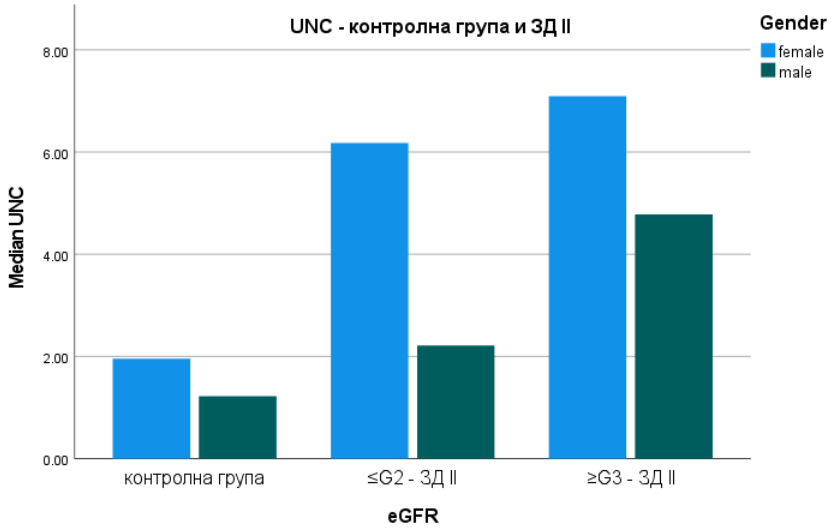


Figure 14. Median UNC in females and males in the control group and in patients with DM II, divided according to eGFR.

The median and interquartile range of uNGAL in patients with DM II with normal renal function is 18.70 (12.00-33.90) ng/ml and in patients with DKD is 49.80 (26.90-104.30) ng/ml. The Kruskal-Wallis test found that the concentration of uNGAL in individual patient subgroups and the control group differed statistically significantly between each other ($\chi 2(^2)$=44.25 p<0.001). Following administration of a post-hoc test, this difference proved to be significant between patients with DKD and the control group (49.80 vs 14.50 ng/ml), U= 418.00 p<0.001 r = -0.611 and between the two patient groups (18.70 vs 49.80 ng/ml), U=370.00 p<0.001 r = -0.484 (Fig. 15).

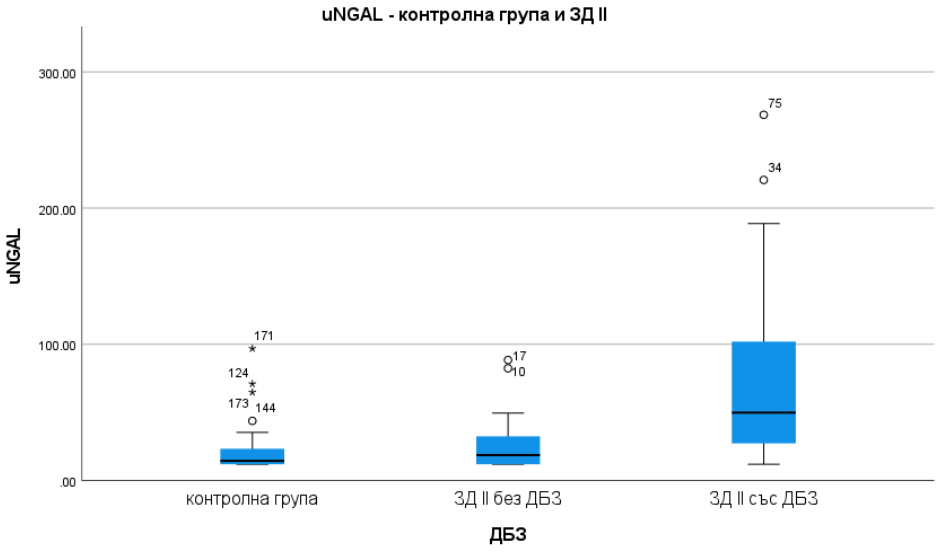


Figure 15. Distribution of uNGAL results in the control group and in patients with DM II with and without DKD.

The median and interquartile range of UNC in patients with DM with normal renal function was 2.26 (1.80-4.07) µg/mmol and in patients with DKD it was 8.54 (4.59-19.84) µg/mmol. The Kruskal-Wallis test found that the concentration of UNC in individual patient subgroups and the control group differed statistically significantly between each other ($\chi^2(2)=69.53$ $p<0.001$). After the post-hoc test, this difference proved to be significant for all comparisons between the groups – the control group and DM II without DKD (2.26 vs 1.75 µg/mmol), U= 578.00 p = 0.002 r = -0.328), the control group and DM II with DKD (8.54 vs 1.75 µg/mmol), U= 181.00 p<0.001 r = -0.752 and between the two patient groups (2.26 vs 8.54 µg/mmol), U=259.00 p<0.001 r= -0.591 (Fig. 16).

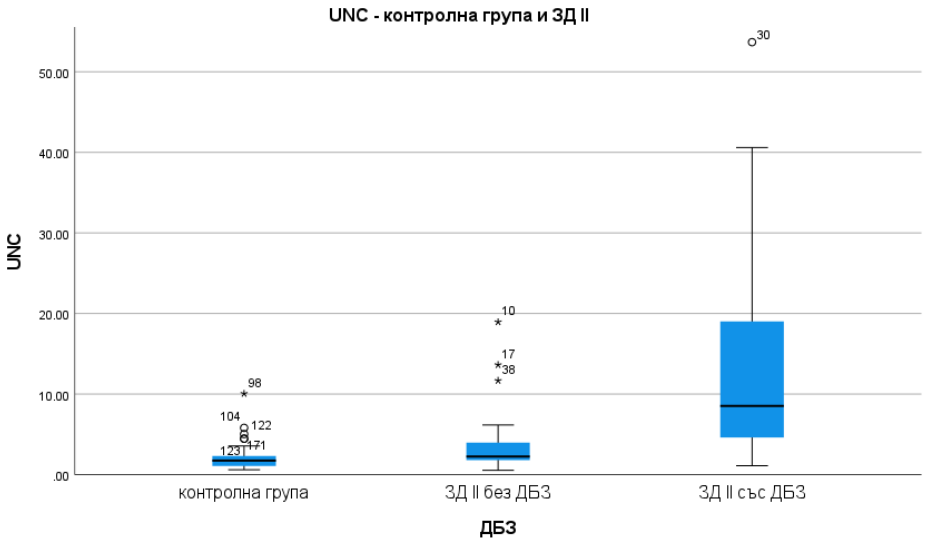


Figure 16. Distribution of UNC results in the control group and in patients with DM II with and without DKD.

In the case of gender division, the medians of UNC in the individual subgroups are as follows: in women – DM II without DKD – 3.74 (2.10-6.07) µg/mmol and DM II with DKD – 8.59 (4.55-19.22) µg/mmol and in men – DM II without DKD – 1.90 (1.65-2.82) µg/mmol and DM II with DKD – 8.22 (4.45-20.67) µg/mmol. The Kruskal-Wallis test found that UNC concentrations in each patient subgroups and in the control group differed statistically significantly between each other, both in females ($\chi^2(2) = 39.48$ $p<0.001$) and in males ($\chi2(^2) =29.07$ $p<0.001$). Following administration of a post-hoc test in women, this difference remained significant for all comparisons between groups. In men, the difference in values between the control group and the patients without DKD did not reach great significance (U=136.00 p=0.019) (α correction at comparison of 3 groups – statistical significance $p\leq0.017$). The UNC values were higher in females compared to males in both subgroups, but a gender difference was significant only in patients with DM without DKD (U=60.00 p = 0.003 r = -0.497) (Fig.17).

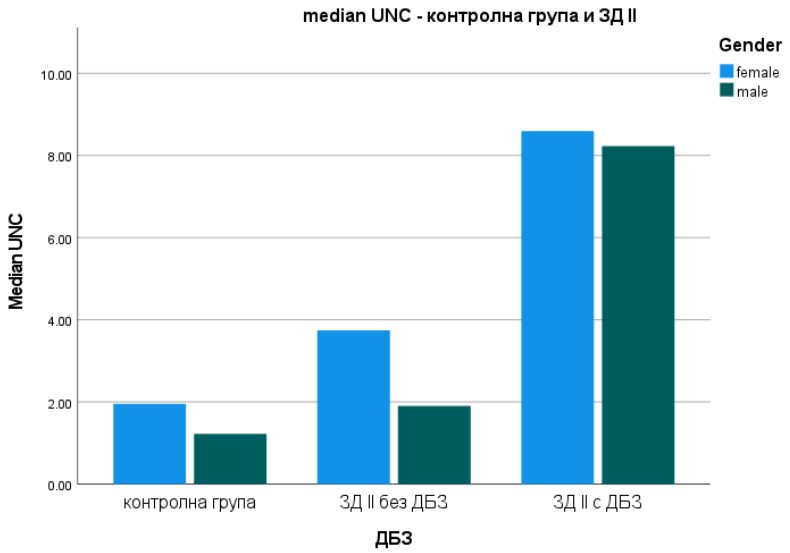


Figure 17. Median UNC in females and males in the control group and in patients with DM II with and without DKD.

The ROC analysis demonstrated good diagnostic effectiveness of uNGAL in differentiating patients with DM II with elevated ACR (A1 vs A2/A3) with AUC-ROC – 0.776. At cut-off 53.30 ng/ml – a value above the defined upper limit of the reference interval, the marker has diagnostic sensitivity and specificity – 47% and 90%, a ratio of positive and negative probability – LR+ 4.67 and LR- 0.59 and a positive and negative predictive value of 84% and 60% in the differentiation of patients with DM II with increased ACR. The diagnostic effectiveness with which a pNGAL value >53.30 ng/ml correctly identifies patients with DM II with ACR >3.0 g/mol is 67% (Fig. 18).

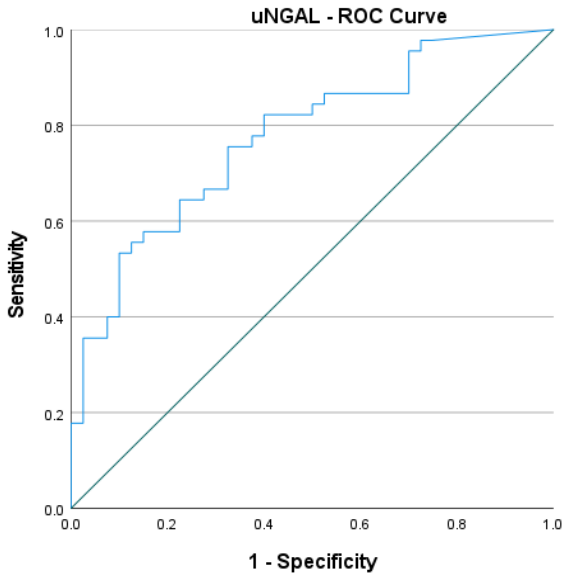


Figure 18. ROC curve of uNGAL – differentiation of patients with MD II with increased ACR

uNGAL has sufficient diagnostic effectiveness to detect patients with DM II with reduced glomerular filtration (eGFR<60ml/min/1.73m²) with AUC-ROC-0.646. In view of this unsatisfactory reliability of the marker, no cut-off values were derived.

uNGAL has good diagnostic effectiveness in distinguishing patients with DKD among persons with DM II (DM without DKD vs DM with DKD) with AUC-ROC – 0.787. At cut-off 53.30 ng/ml – a value above the defined upper limit of the reference interval, the marker has diagnostic sensitivity and specificity – 45% and 94%, a ratio of positive and negative probability – LR+ 7.64 and LR- 0.58, and a positive and negative predictive value of 92% and 53% in the differentiation of patients with DM II with DKD. The diagnostic effectiveness with which a value of uNGAL >53.30 ng/ml is able to correctly identify patients with DM II with DKD is 65% (Fig. 19)

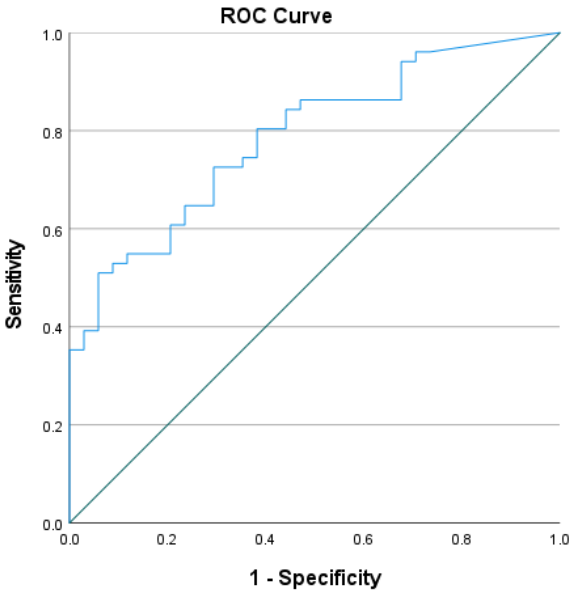


Figure 19. ROC curve – uNGAL in the distinction of patients with DM II with DKD

The UNC ratio shows very good diagnostic accuracy in the differentiation of patients with DM II with albuminuria (A1 vs A2/A3) with AUC-ROC – 0.876. At cut-off – 4.62 µg/mmol, the marker has a diagnostic sensitivity of 82% and a specificity of 80%. When separated by sex, the UNC marker demonstrated AUC-ROC in females – 0.845 and in males – 0.904. The sex-differentiated cut-off values for UNC in identifying patients with increased ACR among subjects with DM II are: in women – 6.87 µg/mmol (diagnostic sensitivity and specificity – 75% and 86%) and in men – 3.72 µg/mmol (diagnostic sensitivity and specificity – 88% and 95%). These determined sex-differentiated cut-off values for differentiation of patients with DM II with increased ACR have a ratio of positive and negative probability – in women – LR+ 5.24 and LR- 0.29 and in men – LR+ 16.64 and LR- 0.12; the positive and negative predictive value – in women – 88% and 72%, and in men – 94% and 90% and diagnostic effectiveness in women – 80% and in men – 92% (Fig. 20).

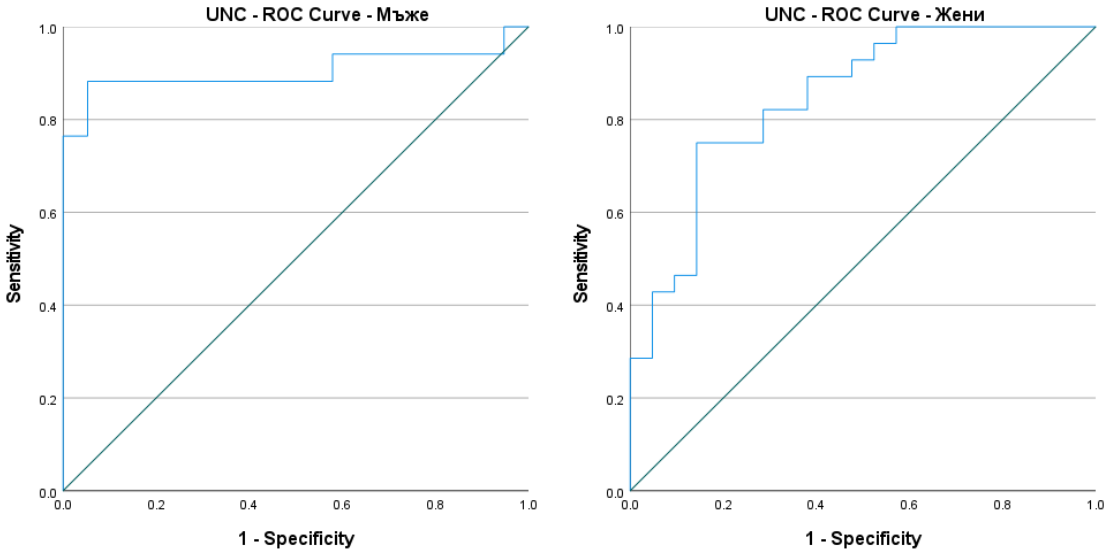


Figure 20. ROC curves of UNC – in females and males, distinguishing patients with DM II with increased ACR

UNC has very good diagnostic effectiveness in distinguishing patients with DKD among persons with DM II (DM without DKD vs DM with DKD) with AUC-ROC – 0.851. At cut-off – 4.62 μg/mmol, the marker has a diagnostic sensitivity of 75% and a specificity of 79%. In division by sex, UNC demonstrated AUC-ROC in females: 0.782, and males – 0.892 in the identification of subjects with renal impairment among patients with DM II. The sex-differentiated cut-off values for DKD of UNC are: in women – 6.87 μg/mmol (diagnostic sensitivity and specificity – 64% and 81%) and in men – 3.72 μg/mmol (diagnostic sensitivity and specificity – 83% and 94%). These sex-differentiated cut-off values for DKD have a ratio of positive and negative probability in women – LR+ 3.38 and LR- 0.44 and in men – LR+ 14.88 and LR- 0.18, positive and negative predictive value in women – 88% and 52% and in men – 94% and 85% and diagnostic effectiveness in women – 69% and in men – 89% (Fig. 21)

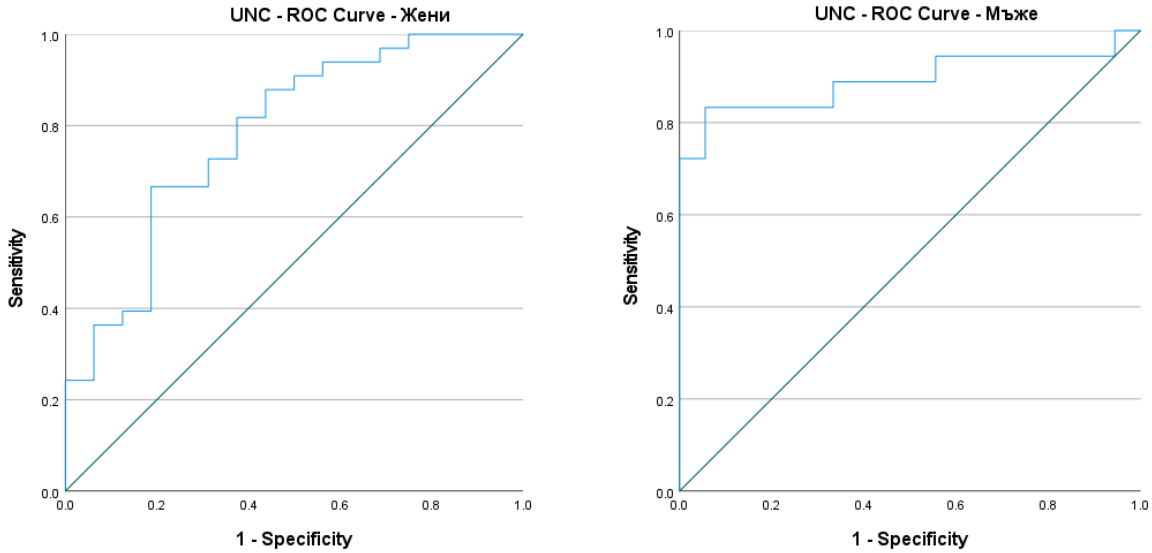


Figure 21. ROC curves: UNC – women and men, distinguishing persons with DM II with DKD

UNC demonstrated poor diagnostic effectiveness in identifying patients with DM II with reduced glomerular filtration (eGFR<60ml/min/1.73m²) (AUC-ROC – 0.578) and no cut-off values were determined.

The statistical processing of the data showed that pNGAL is an appropriate marker for differentiating patients with DM II with reduced glomerular filtration, while uNGAL and UNC are appropriate for identifying those with increased ACR and for detecting patients with DM II with DKD. UNC showed better diagnostic reliability than uNGAL in distinguishing patients with DM II with albuminuria and with DKD (Table 10).

Table 10. Cut-off values for renal impairment in patients with DM II

indicator	criteria	cut-off	N	TP	TN	FP	FN	DSen	DSpec
pNGAL	eGFR<60ml/min/1.73m²	121.65 ng/ml	72	12	43	8	9	57%	84%
uNGAL	ACR>3 g/mol	53.30 ng/ml	85	21	36	4	24	47%	90%
uNGAL	ACR>3 g/mol and/or eGFR<60ml/min/1.73m²	53.30 ng/ml	85	23	32	2	28	45%	94%
UNC – women	ACR>3 g/mol	6.87 µg/mmol	49	21	18	3	7	75%	86%
UNC – men	ACR>3 g/mol	3.72 µg/mmol	36	15	18	1	2	88%	95%
UNC – women	ACR>3 g/mol and/or eGFR<60ml/min/1.73m²	6.87 µg/mmol	49	21	13	3	12	64%	81%
UNC – men	ACR>3 g/mol and/or eGFR<60ml/min/1.73m²	3.72 µg/mmol	36	15	17	1	3	83%	94%

Abbreviations: TP – true positive, TN – true negative, FP – false positive, FN – false negative, DSen – diagnostic sensitivity, DSpec – diagnostic specificity.

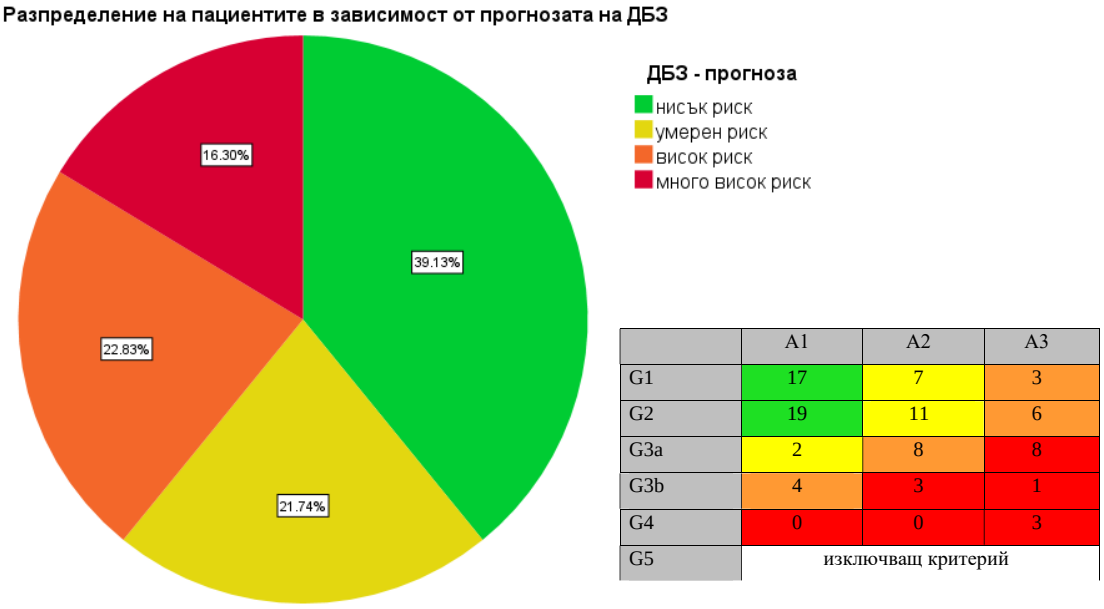
From the data thus obtained, it can be summarized that UNC shows better diagnostic effectiveness than uNGAL in distinguishing patients with DM II with renal impairment, which is why it is preferred in assessing the role of the combined application of two markers – pNGAL and UNC. To determine the significance of pNGAL and UNC for diagnosis of DKD in patients with DM II, defined as ACR>3g/mol and/or eGFR<60ml/min/1.73m², a logistic regression analysis was conducted. When both markers are reported simultaneously, they significantly predict the presence of DKD - $\chi 2$ = 20.405, df =2, p <0.01, but the independent variable pNGAL (p=0.503) does not contribute statistically significantly and is excluded. The reduced regression model, in which only UNC is included, is statistically significant and significantly predicts the presence of DKD - $\chi 2 = 27.575$, df = 1, p < 0.001. The model explained between 28% (Cox & Snell R^2) and 38% (Nadelkerkes R^2) of the dispersion and correctly diagnosed 78% of patients with DM II in accordance with the presence of renal impairment (79% of patients without DKD and 75% of patients with DKD). The exponent of the regression coefficient Exp(B) demonstrates that the increase in UNC by 1 µg/mmol increases the chance that a patient with DM II will have DKD by 1.29 times. After grouping by sex and repetition of the analysis, the regression model was also found to be statistically significant and

independent of pNGAL, with UNC significantly predicting the presence of DKD among patients with DM II (women: $\chi 2 = 9.017$, $p=0.003$ and men: $\chi 2 = 23.788$, $p<0.001$), whereas the increase of UNC by 1 μg/mmol in women increased the probability of DKD 1.16-fold and in men 2.40-fold. The regression model that uses only pNGAL to diagnose DKD among patients with DM II is not significantly ($p= 0.121$), and the one that uses only uNGAL is of lesser priority ($\chi 2 = 22.845$, $p<0.001$).

It is assessed to what extent the determined cut-off value of pNGAL as a marker for reduced glomerular filtration can improve the diagnostic effectiveness of sex-differentiated cut-off value of UNC for DKD. When both cut-off values are applied simultaneously for the detection of DKD, they show the diagnostic sensitivity and specificity – 89% and 73%, the ratio of positive and negative probability – LR+ 3.35 and LR- 0.15 and the positive and negative predictive value – 84% and 81%. The model where cut-off values of pNGAL >121.65 and/or UNC in women > 6.87 μg/mmol and in men > 3.72 μg/mmol are applied simultaneously, has diagnostic effectiveness in identifying patients with DM II with DKD 83% (vs 78% – total diagnostic effectiveness of UNC). From the data thus obtained, it can be summarized that pNGAL improves, but not significantly, the prognostic value of UNC for the detection of patients with DKD among persons with DM II.

5. Diagnostic reliability of NGAL as a marker for the progression of DKD in DM II

According to the guidelines of KDIGO 2020, patients with DM II are classified according to severity and prognosis of DKD, according to the values of ACR and eGFR. Patients were divided into 4 groups: low risk (without DKD) – 36 (39.1%), moderate risk – 20 (21.7%), with high risk – 21 (22.8%) and with very high risk – 15 (16.3%) patients (Fig. 22).



	A1	A2	A3
G1	17	7	3
G2	19	11	6
G3a	2	8	8
G3b	4	3	1
G4	0	0	3
G5	изключващ критерий		

Figure 22. Distribution of patients according to the presence and prognosis of DKD

Since poor glycaemic control is a major factor in the development and progression of DKD, its relationship to the NGAL levels in plasma and urine has been evaluated. Depending on glycaemic control, patients' results were divided into two subgroups, with the defined demarcation value being HbA1c – 7.5%. The characteristics of the patients in the groups divided by the presence and prognosis of DKD, are presented in Table 11 (Table11)

Table 11. Characteristics of the groups of patients with DM II

Category	Criteria	Mean±SD	N (women:men)	Age
low risk	ACR (g/mol) eGFR (ml/min/1.73m²)	1.13±0.50 89.03±15.50	36 (16:20)	56.58±11.95
moderate risk	ACR (g/mol) eGFR (ml/min/1.73m²)	10.10±7.88 80.75±20.76	20 (16:4)	53.15±16.43
high risk	ACR (g/mol) eGFR (ml/min/1.73m²)	47.85 ±80.99 60.90±19.71	21(12:9)	63.62 ±14.37
very high risk	ACR (g/mol) eGFR (ml/min/1.73m²)	96.31±92.86 41.40±13.53	15 (7:8)	61.73±16.86
good control	HbA1c ≤7.5 %	6.54±0.69	20 (12:18)	61.40±13.38
poor control	HbA1c >7.5 %	10.34±1.92	54 (29:25)	54.76±14.30

pNGAL has the following values for median and interquartile range in each patient subgroups, divided according to DKD prognosis – low risk – 70.45 (53.42-93.80) ng/ml, moderate risk – 75.55 (51.60-92.65) ng/ml, high risk – 114.65 (62.40-165.58) ng/ml and very high risk – 121.80 (74.80-191.15) ng/ml. The Kruskal-Wallis test found a statistically significant difference in pNGAL concentration between the subgroups of patients ($\chi 2(3) = 8.715 p=0.033$). After the post-hoc test, this difference did not reach statistical significance for each comparison (Bonferroni correction for α in comparison of 4 groups – statistical significance $p \leq 0.008$) (Fig. 23).

The median and interquartile range of pNGAL in the two subgroups separated by HbA1c are: with good control – 87.60 (58.62-165.00) ng/ml and with poor control – 73.80 (54.32-122.27) ng/ml. The pNGAL values in the control group and in the subgroups of patients, separated by glycaemic control, did not differ significantly between each other ($\chi 2(^2) = 3.547 p=0.170$). ROC analysis demonstrated that pNGAL has poor diagnostic reliability in identifying patients with DM II with poor glycaemic control (AUC-ROC-0.488).

In order to investigate the association between the pNGAL values and the tested indicators for renal impairment and for the control of DM, the Spearman rho rank correlation coefficient was used. The correlation analysis showed a statistically significant positive correlation between pNGAL with the concentration of urea and creatinine in plasma (rho= 0.483 p <0.001 and rho=0.477 p <0.001) and a negative correlation between pNGAL with eGFR (rho= -0.460 p <0.001). There was no significant correlation between pNGAL and ACR as well as with the markers for metabolic and glycaemic control (Table 12).

Table 12. Correlation dependence of pNGAL (Spearman's rho)

		UN	Creat	eGFR	Chol	TG	HDL	LDL	HbA1c	ACR	uNGAL	UNC
pNGAL	rho	0.483	0.477	-0.460	-0.109	0.010	-0.150	-0.118	-0.206	0.188	0.202	0.128
	p	<0.001**	<0.001**	<0.001**	0.364	0.935	0.219	0.334	0.109	0.116	0.106	0.311

** statistically significant correlation p<0.001

uNGAL has the following values for median and interquartile range in individual patient subgroups, divided according to DKD prognosis – low risk -18.70 (12.00-33.90) ng/ml, moderate risk – 36.50 (24.70-62.65) ng/ml, high risk – 47.00 (15.22-102.15) ng/ml and very high risk – 99.80 (30.20-135.20) ng/ml. The Kruskal-Wallis test found a statistically significant difference in uNGAL concentration between subgroups of patients ($\chi 2(3) = 22.855$, $p<0.001$). Following the post-hoc test, this difference was only significant in the comparisons of the low-risk group – with moderate risk (U=139.00 p = 0.001 r = -0.446), high-risk (U=167.00 p = 0.007 r = -0.373) and very high risk group (U=64.00 p<0.001 r = -0.594) (Bonferroni correction for α in comparison to 4 groups – statistical significance p≤0.008) (Fig. 23).

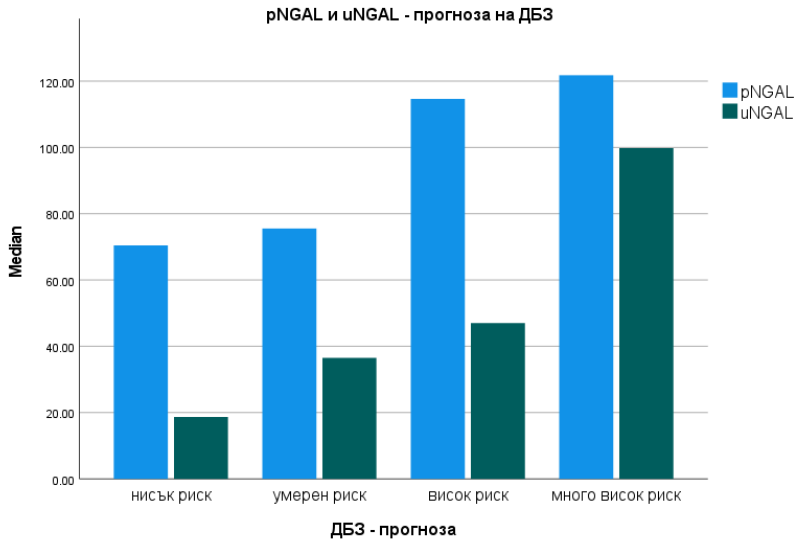


Figure 23. Median pNGAL and uNGAL in patients with DM II divided according to presence and prognosis of DKD

UNC has the following values for median and interquartile range in the individual patient subgroups, divided according to DKD prognosis – low risk – 2.26 (1.79-4.06) µg/mmol, moderate risk – 7.85 (4.48-18.01) µg/mmol, high risk – 6.21 (3.60-33.78) µg/mmol and very high risk – 9.77 (7.34-18.21) µg/mmol. The Kruskal-Wallis test found a statistically significant difference in uNGAL concentration between subgroups of patients ($\chi 2(3)=31.256$, $p<0.001$). After the post-hoc test, this difference was only significant in all comparisons of the low-risk group - with moderate-risk

(U=82.00 p<0.001 r = -0.598), high-risk (U=136.00 p = 0.001 r = -0.454) and with very high-risk (U=41.00 p<0.001 r = -0.663) (Fig. 24).

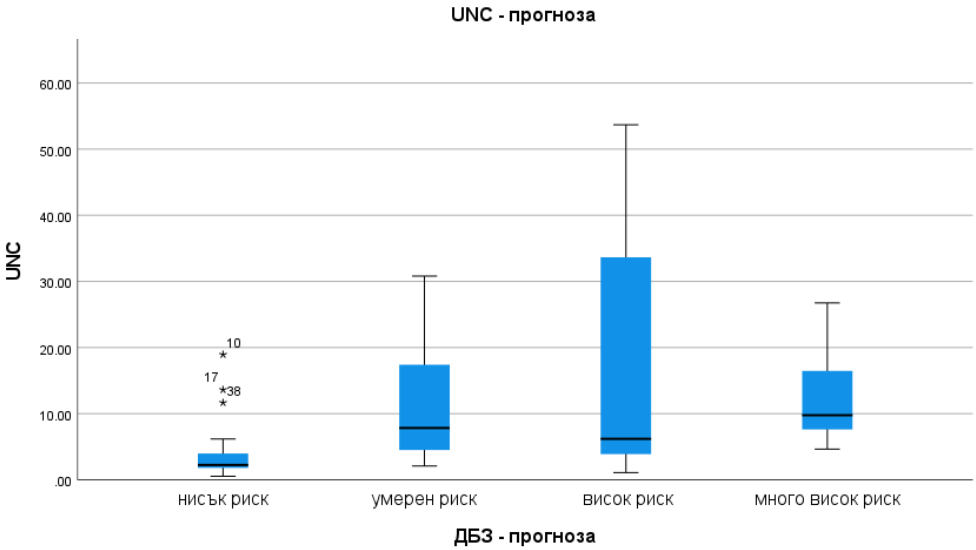


Figure 24. Distribution of UNC results for patients with DM II, separated according to presence and prognosis of DKD

The median and interquartile range of uNGAL in the two subgroups separated by HbA1c are: good control – 27.10 (18.80-49.50) ng/ml and poor control – 32.40 (16.32-60.40) ng/ml. The Mann-Whitney test showed no significant difference in uNGAL values between subgroups of patients (p= 0.861), but the results of uNGAL in each subgroup were significantly higher than in the control group (good control - U= 262.0 p<0.001 r = -0.385 and poor control - U= 716.0 p<0.001 r = -0.423). uNGAL demonstrated poor diagnostic effectiveness in identifying patients with DM II with poor glycaemic control with AUC-ROC 0.514.

The UNC ratio has a median and interquartile range in a subgroup with good glycaemic control – 3.98 (1.98-10.30) µg/mmol and in a subgroup with poor glycaemic control – 4.86 (2.18-13.63) µg/mmol. There was no significant difference in UNC values between subgroups of patients (p= 0.452), but the UNC results in each subgroup were significantly higher than in the control group (good control - U= 202.0 p<0.001 r = -0.464 and poor control – U=475.0 p<0.001 r = -0.570). UNC demonstrated poor diagnostic effectiveness in identifying patients with DM II with poor glycaemic control with AUC-ROC-0.559.

Spearman rho's correlation analysis showed a statistically significant positive correlation between uNGAL with urinary albumin concentration (AER) and ACR (rho= 0.610 p <0.001 and rho= 0.514 p <0.001) and a negative correlation between uNGAL and eGFR (rho = -0.232 p =0.033). No significant correlation was found between uNGAL and the markers for metabolic and glycemic control of the DM. The UNC ratio correlates significantly positively with AER (rho= 0.492 p <0.001), ACR (rho=

0.692 p <0.001), HbA1C (rho=0.238 p =0.049) and negatively with eGFR (rho = -0.265 p=0.014). No significant correlation was found between UNC and the markers evaluating lipid status (Table 13).

Table 13. Correlation dependence of uNGAL and UNC (Spearman's rho)

		AER	ACR	eGFR	HbA1c	UN	Creat	Chol	TG	HDL	LDL	pNGAL
uNGAL	rho	0.610	0.514	-0.232	0.060	0.056	0.160	0.112	0.026	0.072	0.024	0.202
	p	<0.001**	<0.001**	0.033*	0.624	0.616	0.144	0.318	0.816	0.529	0.833	0.106
UNC	rho	0.492	0.692	-0.265	0.238	0.103	0.138	0.084	0.084	-0.037	-0.067	0.128
	p	<0.001**	<0.001**	0.014 *	0.049	0.349	0.207	0.458	0.455	0.745	0.562	0.311

*statistically significant correlation p<0.05
** statistically significant correlation p<0.05

In order to assess the predictive value of the pNGAL and UNC markers in the classification of patients according to the severity and prognosis of DKD, a discriminatory analysis was conducted. The analysis found that the simultaneous use of the two markers has a better predictive value than using them alone. Both markers are statistically significantly related to the distribution of patients by group. The model using pNGAL and UNC is able to statistically significantly discriminate groups with DKD – λ=0.665 $\chi 2 = 24.904$ p<0.001 η=0.36 and explains 26.8% of the distribution of patients with DM by severity and prognosis of renal impairment. The discriminant function is: D = -1.43+(UNCx0.068) + (pNGALx0.08). This model successfully predicted the distribution of 57% of patients with DM II in the respective groups according to the degree of renal impairment and their prognosis. The model, which uses only pNGAL, uNGAL or only UNC, successfully predicts the distribution of 26%, 42.4% and 49% of patients, respectively, relative to the grade of DKD and its prognosis (Fig. 25).

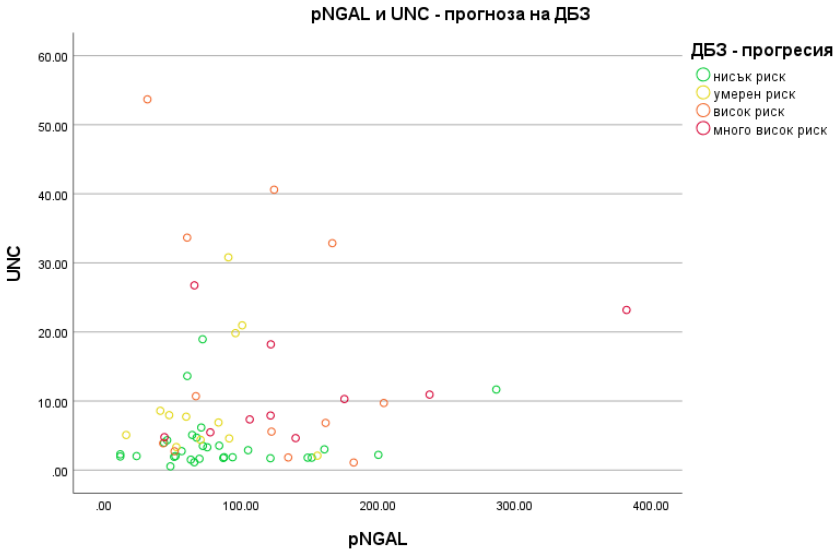


Figure 25. Distribution of pNGAL and UNC results for patients with DM II, separated according to presence and prognosis of DKD

In order to assess the likelihood that pNGAL and UNC can successfully predict the presence of high and very high risk DKD among patients with DM II, a logistic regression analysis was conducted. Patients were divided into two groups, the first group with low and moderate risk and the second group with high and very high risk. A regression model in which pNGAL and UNC are included is statistically significant and significantly predicts the presence of DKD with high and very high risk - χ^2 = 18.558, df =2, p <0.01, and both markers contribute statistically significantly to the correct distribution of patients in both groups. The model explains between 25% (Cox & Snell R^2) and 34% (Nadelkerkes R^2) of the dispersion and correctly classifies 75% of the patients with DM II to the defined groups – 91% of patients in the low/moderate risk group and 48% of patients in the high/very high risk group. The exponent of the regression coefficient Exp(B) demonstrates that the increase in pNGAL by 1ng/ml increases the chance that a patient with DM II will have a DKD with high and very high risk by 1.01 times, and the increase in UNC by 1 µg/mmol increases the probability that a patient with DM II will have a DKD with high and very high risk by 1.09 times (women – 1.07 times, men – 1.14 times). The model, which uses only pNGAL or only UNC correctly classifies 66% and 65% of patients respectively to the low/moderate and high/very high risk groups.

From the data thus obtained, it can be summarized that the simultaneous use of the two markers – pNGAL and UNC achieves a more accurate estimation of the DKD prognosis than when applied alone (Fig. 26).

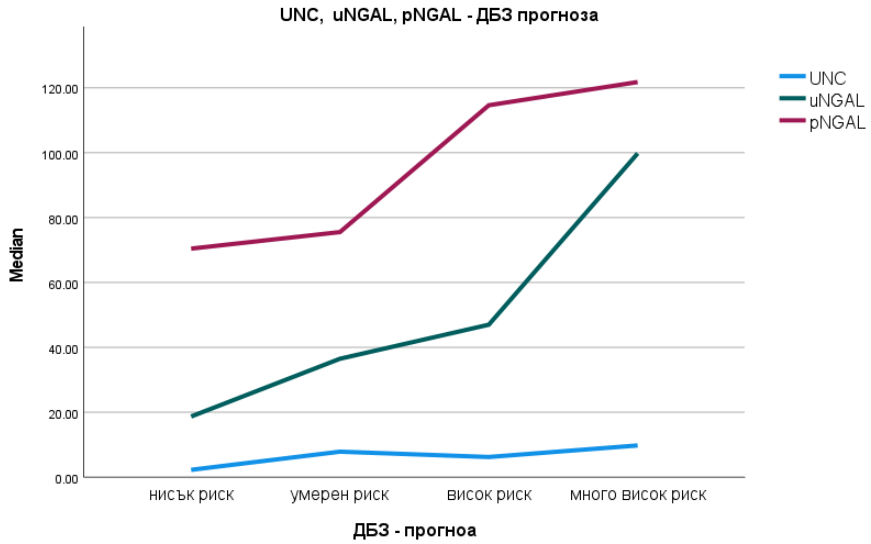


Figure 26. Change in median UNC, uNGAL and pNGAL in DKD progression

Seven of the targeted patients with DM II did not meet the inclusion criteria – in 5 patients, significant leukocytosis was found ($\bar{x}$ - pNGAL=79.06 ng/ml, uNGAL= 248.76 ng/ml and UNC= 47.87 µg/mmol), in one patient – leukocytosis (pNGAL =129.00 ng/ml, uNGAL = 40.80 ng/ml and UNC=4.73 µg/mmol) and in one of them – corticosteroid intake (pNGAL = 375.20 ng/ml, uNGAL

= 31.80 ng/ml and UNC=4.55 µg/mmol). These patients were excluded from statistical data processing (Fig. 27).

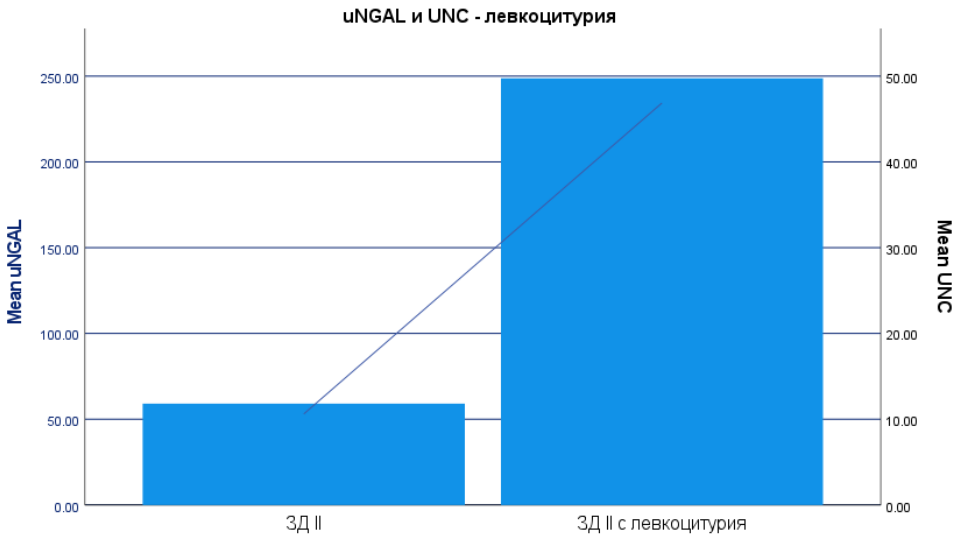


Figure 27. Mean – uNGAL and UNC in patients with DM II with and without leukocyturia

6. Diagnostic reliability of NGAL in the diagnostics of DKD in DM I

78 children with DM I were targeted for participation in the study, but 3 of them had criteria for exclusion from the group. Persons with DM I meeting the criteria included in this study are 75 patients aged 6 to 17 years (13.38±2.82), with an onset of DM from 5 to 14 years ago (7.09 ±2.59). Of the patients included in the group, 44% (N=33) were girls aged 13.24 ±3.13 and 56% (N=42) were boys aged 13.50±2.59. A characteristic of the patient cohort with DM I is presented in Table 14 (Table 14).

In this patient cohort, ACR >30 g/mol was found in 2 of the enrolled children, therefore patient outcomes were divided into only two subgroups: A1 – with ACR <3 g/mol, A2 – with ACR – 3-30 g/mol, whereas subgroup A3 was not formed. A decrease in eGFR <60 ml/min/1.73m² was observed in only one of the enrolled patients, therefore the role of pNGAL in the diagnosis of glomerular hyperfiltration was assessed and the patient cohort was divided into two subgroups according to the eGFR (Bedside Schwartz) and the eGFR (CKD-EPI40) values, with a cut-off value of 90 ml/min/1.73m². Depending on glycaemic control, the results of children with DM I were divided into two subgroups, with the defined demarcation value being HbA1c – 7.5%. Since only one patient showed a decrease in eGFR<60 ml/min/1.73m², who also has pathological albuminuria, it is unnecessary to form groups – DM I without DKD and DM I with DKD, as they are identical to A1 and A2. Cut-off values derived in the present study to identify pathological albuminuria in patients with DM I correspond to cut-off values for DKD (Table 15)

Table 14. Characteristics of the patient group with DM I

Indicator	Patients DM I mean±SD / median(IQR)	Control group <18 yrs. mean±SD /median(IQR)	p
number	75	42	
gender girls:boys (%)	44%:56%	50%:50%	=0.536
years (yrs)	13.39±2.82	12.50±3.69	=0.181
glucose (mmol/l)	10.72 ±5.63	4.69 ±0.71	<0.001
urea (mmol/l)	4.70 ±1.33	3.94 ±1.05	=0.006
creatinine (µmol/l)	57.10 ±13.38	59.66 ±16.22	=0.413
eGFR (ml/min/1.73m²) (Bedside Schwartz)	104.04±20.95	*	*
eGFR (ml/min/1.73m²) (CKD-EPI40)	98.35±16.80	94.11±16.42	=0.318
total cholesterol (mmol/l)	4.48±1.01	4.28±0.96	=0.420
triglycerides (mmol/l)	1.24 ±1.41	0.95 ±0.46	=0.364
HDL-cholesterol (mmol/l)	1.43±0.42	*	*
LDL-cholesterol (mmol/l)	2.53±0.86	*	*
AER (mg/l)	38.21 ±110.07	*	*
HbA1c (%)	8.47 ±2.10	*	*
ACR (g/mol)	4.75 ±14.35	*	*
BMI	21.06 ±4.46	*	*
BMI (patient)/BMI (targeted age)	1.12 ±0.21	*	*
duration of diabetes	7.09 ±2.59	*	*
pNGAL (ng/ml)	66.38 ±31.34 / 58.20(33.40)	62.55±20.16 / 66.15(28.45)	=0.754
uNGAL (ng/ml)	42.46±97.88 / 13.00(15.00)	19.96±13.93 / 13.70(10.35)	=0.683
UNC (µg/mmol)	5.11±8.69 / 1.94 (2.14)	1.72±0.90 / 1.47(1.06)	=0.022

Table 15. Characteristics of the groups of patients with DM I

Category	Criteria	Mean±SD	N(%)	Age
A1	ACR <3 g/mol	0.84±0.54	53 (72%)	13.19 ±2.68
A2.	ACR-3 – 30 g/mol	7.00±5.11	19 (25%)	13.84 ±3.08
A3(not formed)	ACR >30 g/mol	87.22±5.75	2 (3%)	16.50 ±0.71
eGFR(Bedside Schwartz) ≥90	eGFR≥90 ml/min/1.73m²	110.62±18.39	15 (21%)	13.07±2.87
eGFR(Bedside Schwartz)<90	eGFR <90 ml/min/1.73m²	79.93±8.55	55 (79%)	13.38±2.81
eGFR(CKD-EPI40) ≥90	eGFR≥90 ml/min/1.73m²	107.52±8.59	24 (32%)	12.96±2.90
eGFR(CKD-EPI40) <90	eGFR <90 ml/min/1.73m²	77.54±9.41	50 (68%)	13.52±2.78
good control	HbA1c ≤7.5 %	6.77±0.63	33 (45%)	13.00±2.65
poor control	HbA1c >7.5 %	9.84±1.86	41 (55%)	13.61±2.94

eGFR in patients with DM I was calculated using the established formula of 'Bedside Schwartz' – $GFR_{(Bedside\ Schwartz)}$ and the alternative formula CKD-EPI40 - $eGFR_{(CKD\text{-}EPI40)}$. In order to establish the linear regression between CKD-EPI40 and the Bedside Schwartz formula, a single regression analysis was conducted which showed that $eGFR_{(Bedside\ Schwartz)}$ can significantly predict $eGFR_{(CKD\text{-}EPI40)}$, $F(1.75) = 101.19$ $p<0.001$. The regression constant ($\alpha=33.97$ $p<0.001$) and the regression coefficient ($b= 0.613$ $p < 0.001$) were statistically significant. The equation found for the relationship between the results of the two formulae is: $eGFR_{(CKD\text{-}EPI40)} = 33.97+0.613 x eGFR_{(Bedside\ Schwartz)}$. The value of the adjusted determination coefficient (adjusted R^2) is 0.592, indicating that 59% of the changes in $eGFR_{(CKD\text{-}EPI40)}$ can be explained by the presented regression model, i.e. by $eGFR_{(Bedside\ Schwartz)}$. According to Cohen (Cohen, 1988) this is much larger than the typical magnitude of the effect (Fig. 25).

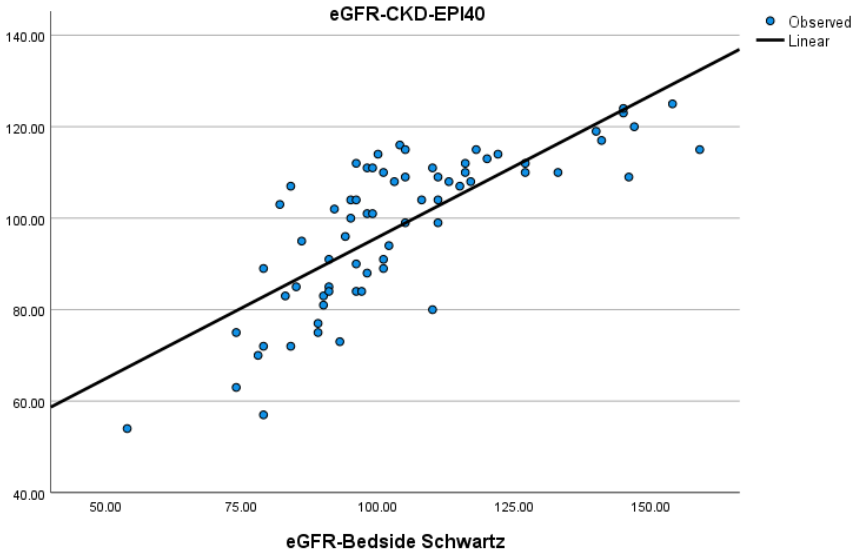


Figure 28. Regression line representing the linear relationship between $eGFR_{(CKD\text{-}EPI40)}$ and $eGFR_{(Bedside\ Schwartz)}$

The statistical check for normal distribution of results for pNGAL, uNGAL and UNC across the cohort of patients with DM I and across subgroups showed the lack of Gaussian distribution. The Shapiro-Wilk's test ($p<0.05$) demonstrated coefficients for asymmetry and excess with z-value >1.96, which rejected the null hypothesis of normal distribution for all three indicators in each of the examined subgroups. The subsequent statistical processing of the data was carried out using non-parametric methods of analysis.

6.1. pNGAL as a marker in the diagnostics of DKD in patients with DM I

The median and interquartile range of pNGAL across the patient group was 58.20 (46.70-80.10) ng/ml. TheMann-Whitney test found no statistically significant difference in pNGAL concentration between patients with DM I and their respective control group ($U=1369$ $p=0.754$). The median and interquartile range in the two subgroups separated by ACR were: A1 - 57.75 (49.45-77.30) ng/ml

and A2 - 66.50 (35.35-124.13) ng/ml. The Kruskal-Wallis test found no statistically significant difference in pNGAL concentration between the control group and individual patient subgroups separated by ACR ($\chi^2(2)=0.308$ p=0.857).

The median and interquartile range of pNGAL in the two subgroups separated by eGFR (Bedside Schwartz) were: for eGFR (Bedside Schwartz) ≥90 e 56.50 (47.00-74.95) ng/ml and for eGFR (Bedside Schwartz) <90 e 80.60 (41.75-121.75) ng/ml. The pNGAL values in the eGFR(Bedside Schwartz) group <90 ml/min/1.73m² were higher than in the eGFR(Bedside Schwartz) patients ≥90, but the difference did not reach great significance (U= 288.5 p=0.110). When applying the alternative formula – eGFR (CKD-EPI40) to classify patient outcomes, a median pNGAL was found in the eGFR(CKD-EPI40) group ≥90 – 53.95 (41.63-67.30) ng/ml and in the eGFR(CKD-EPI40) group < 90 - 77.10 (53.90-111.60) ng/ml. The Kruskal-Wallis test found that the concentration of pNGAL in the patient subgroups thus defined and the control group differed statistically significantly between each other ($\chi2(^2)$ = 10.082, p=0.006). Following administration of a post-hoc test, this difference proved to be significant only between the two patient subgroups (53.95 vs 77.10 ng/ml), U=317.0 p=0.004 r = -0.34 (Fig. 29).

The median and interquartile range of pNGAL in the two subgroups separated by HbA1c were: good control -59.00 (50.7-93.9) ng/ml and poor control – 54.90 (41.5-77.1) ng/ml. The values of pNGAL between subgroups of patients divided by glycaemic control did not differ significantly from each other and from the control group ($\chi^2(2)$ =0.716 p= 0.699).

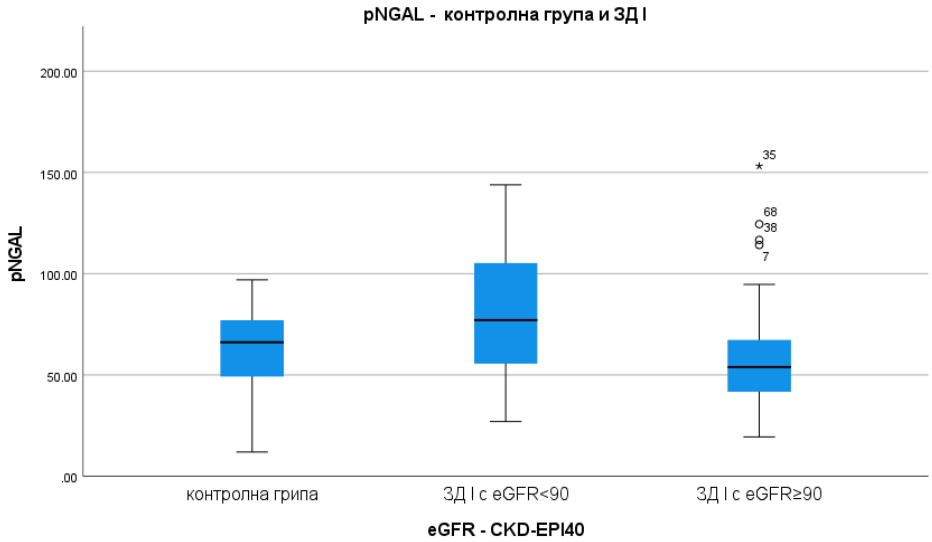


Figure 29. Distribution of pNGAL scores in the control group and in patients with DM I divided by eGFR(CKD-EPI40)

Spearman rho correlation analysis showed a statistically significant negative correlation between pNGAL and eGFR(CKD-EPI40) (rho = -0.324 p=0.006), but not with eGFR(Bedside Schwartz). There was no significant correlation between pNGAL and ACR as well as with the markers for metabolic and

glycaemic control and with the duration of DM I (Table 16).

Table 16. Correlation dependence of pNGAL (Spearman's rho)

		eGFR		Urea	Creat	Chol	TG	HDL	LDL	BMI	HbA1c	ACR
		CKDEPI	Schwartz									
pNGAL	rho	-0.324	-0.197	-0.214	0.221	0.047	-0.173	0.118	0.021	0.116	-0.148	-0.180
	p	=0.006 *	=0.110	0.076	=0.064	=0.698	=0.152	=0.330	=0.864	=0.354	=0.228	=0.136

* statistically significant correlation p<0.05

The ROC analysis demonstrated good diagnostic effectiveness of pNGAL in differentiating DM I patients with eGFR $_{(CKD\text{-}EPI40)}$ < 90ml/min/1.73m², with AUC-ROC-0.713. At cut-off of 96.80 ng/ml, pNGAL has diagnostic sensitivity and specificity of 30% and 92%, ratio of positive and negative probability – LR+ 3.66 and LR- 0.76, positive and negative predictive value – 64% and 73% and diagnostic efficiency of 72% in the distinction of patients with DM I with slightly reduced glomerular filtration (Fig. 30).

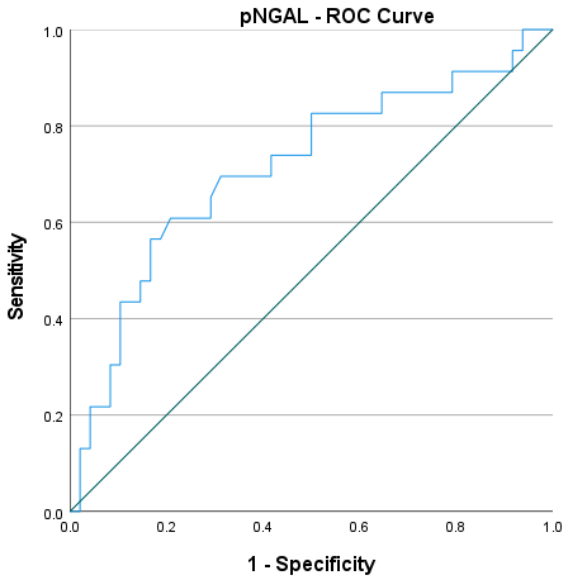


Figure 30. ROC curve of pNGAL in distinguishing patients with DM I with eGFR <90 ml/min/1.73m²

6.2.uNGAL and UNC as markers in the diagnostics of DKD in patients with DM I

The median and interquartile range of uNGAL and UNC across the patient group were 13.00 (12.00-27.00) ng/ml and 1.94 (1.18-3.32) µg/mmol. There was no significant difference in uNGAL concentration between patients with DM I and the control group (U=1431 p= 0.683). Significantly higher UNC scores were observed in patients with DM I compared to the control group (1.94 vs 1.47 µg/mmol) U=1107.5 p=0.022 r = -0.22.

The median and interquartile range of uNGAL in the two subgroups separated by ACR were: A1 – 12.00 (12.00-19.57) ng/ml and A2 – 41.4 (17.60-83.70) ng/ml. The Mann-Whitney test found a statistically significant difference in uNGAL concentration between subgroups of patients with DM I, U= 179.0, p<0.001 r = -0.517. Only patients from subgroup A2 (41.4 vs 13.70 ng/ml), U= 184.5 p<0.001 r = -0.431, had significantly higher scores compared to the control group. (Fig. 31).

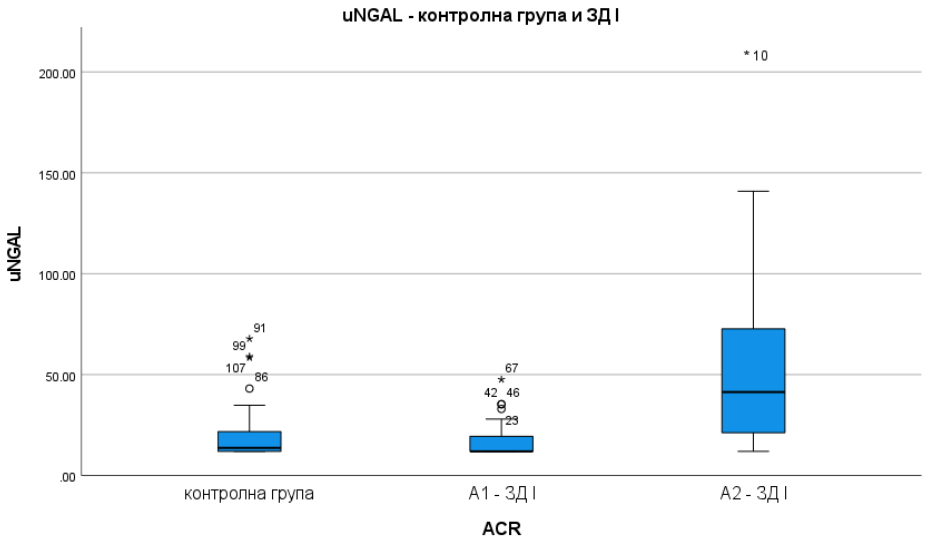


Figure 31. Distribution of uNGAL results in the control group and in patients with DM I, divided according to ACR

The UNC ratio has the following values for median and interquartile range in individual patient subgroups: A1 – 1.64 (0.98-2.54) µg/mmol and A2 – 8.49 (2.50-15.32) µg/mmol. The Kruskal-Wallis test found that the UNC values in the control group and in the individual subgroups separated by ACR differed significantly between each other ($\chi 2(^2)$ =26.35, p<0.001). Following administration of a post-hoc test, this difference proved to be significant for all comparisons between the groups except for A1 and the control group. The Mann-Whitney test found a statistically significant difference in UNC values between the two patient subgroups (1.64 vs 8.49 µg/mmol), U= 139.0p<0.001 r = -0.547. Only patients in A2 (8.49 vs 1.47 µg/mmol), U=84.0 p<0.001 r=-0.626 had significantly higher scores compared to the control group. (Fig. 32).

There was no statistically significant difference in uNGAL concentration between the control group and individual patient subgroups separated by eGFR $_{(Bedside\ Schwartz)}$ ($\chi^2(2) = 0.492$, p=0.782) and by eGFR $_{(CKD\text{-}EPI40)}$ ($\chi^2(2)$ =2.115, p= 0.347). The Kruskal-Wallis test found that UNC values in the control group and in the individual subgroups separated by eGFR$_{(Bedside\ Schwartz)}$ as well as by eGFR $_{(CKD\text{-}EPI40)}$ differed from each other, but this difference did not reach statistical significance for each comparison after the post-hoc test (Bonferroni correction for α in comparison with 3 groups – statistical significance p≤0.017).

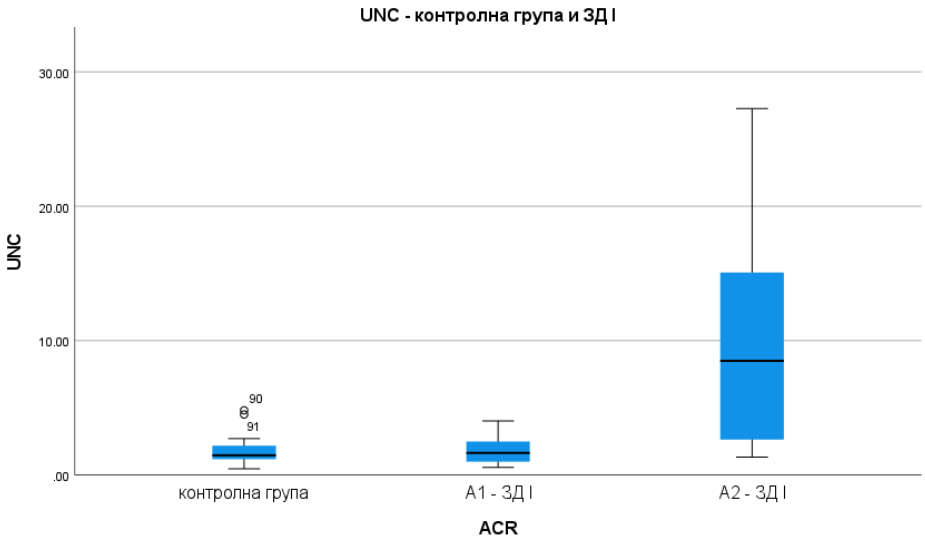


Figure 32. Distribution of UNC results in the control group and in patients with DM I, divided according to ACR

The median and interquartile range of uNGAL in the two subgroups of patients separated by HbA1c were: good control –12.00 (12.00-20.90) ng/ml and poor control – 16.10 (12.00-39.90) ng/ml. The Kruskal-Wallis test found no statistically significant difference in uNGAL concentration between the control group and individual patient subgroups separated by HbA1c ($\chi^2(2) = 4.661 p=0.097$).

The UNC ratio has a median in the good glycaemic control subgroup – 1.71 (0.99-2.39) µg/mmol and in the poor glycaemic control subgroup – 2.79 (1.55-8.25) µg/mmol. The Kruskal-Wallis test showed a statistically significant difference between control and patient groups ($\chi^2(2) = 12.86 p=0.002$). After administration of a post-hoc test, it was found that patients with poor glycaemic control had significantly higher scores compared to both the control group and those with good control of DM I (2.79 vs 1.47 µg/mmol), U= 468.0 $p<0.001$ r = -0.369 and (2.79 vs 1.71 µg/mmol), U= 396.0 p=0.006 r = -0.326 (Fig. 33).

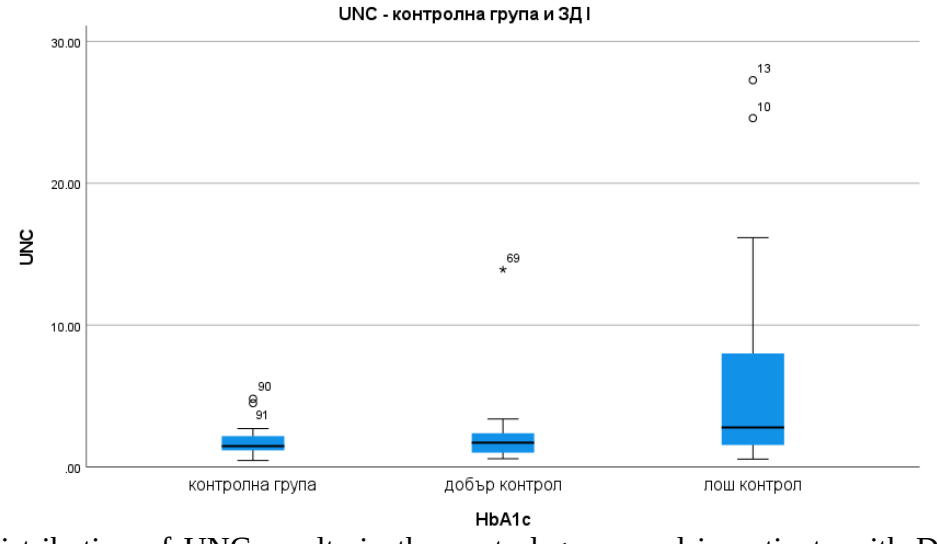


Figure 33. Distribution of UNC results in the control group and in patients with DM I, divided according to HbA1c

Spearman's rho correlation analysis showed a statistically significant positive correlation between uNGAL with AER, ACR, HbA1c, BMI, triglycerides and age (rho= 0.408 p <0.001, rho= 0.389 p <0.001, rho=0.255 p = 0.031, rho= 0.273 p =0.025, rho= 0.262 p =0.027 and rho=0.250 p =0.033). UNC positively correlates significantly with ACR, AER, HbA1c and triglycerides (rho=0.641 p <0.001, rho=0.250 p =0.033, rho= 0.435 p <0.001, rho= 0.396p <0.001). There was no significant correlation of both uNGAL and UNC with $eGFR_{(Bedside\ Schwartz)}$ and $eGFR_{(CKD\text{-}EPI40)}$, as well as with the duration of MD I (Table 17).

Table 17. Correlation dependence of uNGAL and UNC (Spearman's rho)

		AER	ACR	HbA1c	Chol	TG	HDL	LDL	BMI	pNGAL	eGFR	
											Schwartz	CKDEPI
uNGAL	rho	0.408	0.389	0.255	0.142	0.262	-0.089	0.066	0.273	0.042	0.036	0.115
	p	<0.001**	<0.001**	=0.031 *	=0.237	=0.027*	=0.459	=0.584	=0.025*	=0.732	=0.771	=0.336
UNC	rho	0.250	0.641	0.435	0.163	0.396	-0.152	0.081	0.178	-0.172	0.081	0.194
	p	=0.033*	<0.001*	<0.001**	=0.176	<0.001**	=0.207	=0.500	=0.149	=0.157	=0.510	=0.102

*statistically significant correlation p<0.05
** statistically significant correlation p<0.01

The ROC analysis demonstrated very good diagnostic effectiveness of uNGAL in differentiating patients with DM I with elevated ACR (A1 vs A2/A3) with AUC-ROC – 0.836. At cut-off- 47.85 ng/ml, a value above the defined upper limit of reference, uNGAL has a diagnostic sensitivity and specificity of 52% and 100%, respectively, a ratio of positive and negative probability – LR+ >52.00 and LR- 0.48, and positive and negative predictive value – 100% and 84%, respectively, in distinguishing patients with DM I with ACR>3 g/mol. The diagnostic effectiveness, with which the uNGAL value >47.85 ng/ml correctly identifies patients with DM I with albuminuria, is 86%. The cut-off value thus determined can be taken as a borderline value for DKD in children with DM I (Fig. 34).

The UNC ratio shows very good diagnostic accuracy in the differentiation of patients with DM I with albuminuria (A1 vs A2/A3) with AUC-ROC – 0.873. The cut-off value, which has the best diagnostic characteristics in the differentiation of patients with MD I with ACR >3 g/mol is 3.86 µg/mmol, with diagnostic sensitivity and specificity – 67% and 98%, ratio of positive and negative probability – LR+ 35.10 and LR- 0.34, and positive and negative predictive value – 93% and 88%. The diagnostic effectiveness, with which a UNC value of >3.86 µg/mmol was able to correctly identify patients with DM I with albuminuria, was 89%. The cut-off value thus determined can be taken as a borderline value for DKD in children with DM I (Fig. 34).

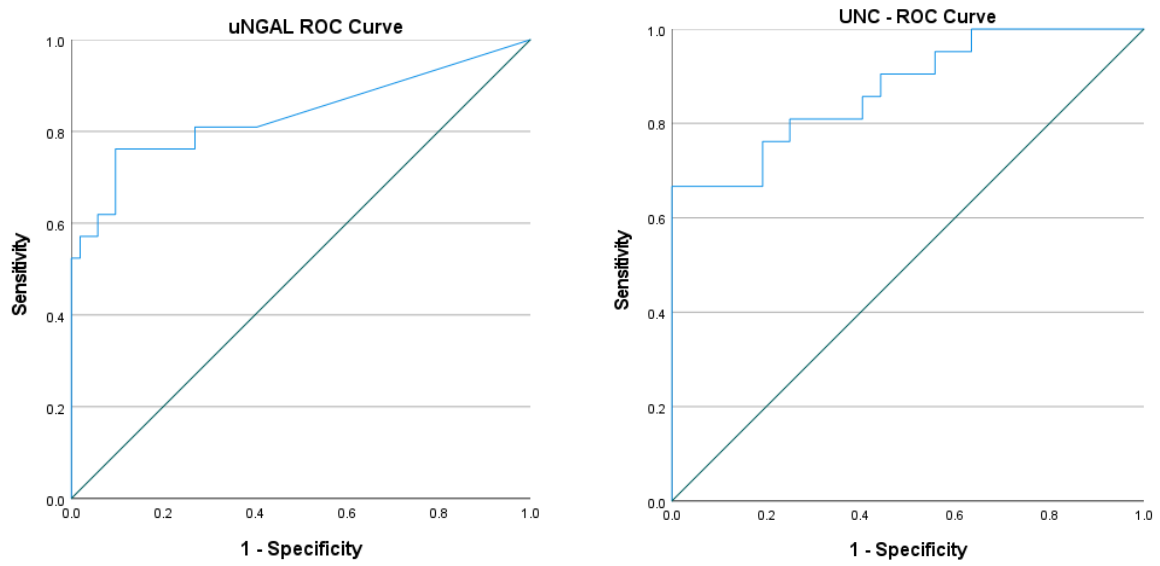


Figure 34. ROC curves of uNGAL and UNC in differentiating patients with MD I with elevated ACR

The statistical processing of the data showed that pNGAL is an appropriate marker for differentiating patients with DM I with slightly decreased glomerular filtration, while uNGAL and UNC are suitable for identifying patients with increased ACR and for detecting DKD among patients with DM I. The cut-off values for renal impairment in patients with DM I are presented in Table 18 (Table 18).

Table 18. Summary of cut-off values in patients with DM I, derived in the present study

indicator	criteria	cut-off	N	TP	TN	FP	FN	DSen	DSpec
pNGAL	eGFR<90ml/min/1.73m²	96.80 ng/ml	71	7	44	4	16	30%	92%
uNGAL	ACR>3 g/mol	47.85 ng/ml	73	11	52	0	10	52%	100%
UNC	ACR>3 g/mol	3.86 µg/mmol	73	14	51	1	7	67%	98%

Abbreviations: TP – true positive, TN – true negative, FP – false positive, FN – false negative, DSen – diagnostic sensitivity, DSpec – diagnostic specificity

From the data thus obtained, it can be summarized that UNC shows better diagnostic effectiveness than uNGAL in distinguishing patients with DM I with renal impairment, which is why it is preferred in assessing the role of the combined application of two markers – pNGAL and UNC. To determine the significance of pNGAL and UNC for diagnosis of DKD in patients with DM I, defined as ACR>3g/mol, a logistic regression analysis was conducted. When both markers are reported simultaneously, they significantly predict the presence of DKD – χ^2= 44.285 df= ^{2}, p<0.01, whereas the independent variable pNGAL (p=0.037) contributes statistically significantly, but does not significantly improve the model.The reduced regression model, in which only UNC is included, is statistically significant and significantly predicts the presence of DKD - χ^2 = 43.505, df = 1, p < 0.001. The model explained between 45% (Cox & Snell R^2) and 64% (Nadelkerkes R^2) of the dispersion and

correctly diagnosed 90% of patients with DM I given the presence of renal impairment (99% of patients without DKD and 67% of patients with DKD). The exponent of the regression coefficient Exp(B) demonstrates that the increase in UNC by 1 µg/mmol increases the chance that a patient with DM I will have DKD by 2.1 times. The regression model that uses only pNGAL to diagnose DKD among patients with DM I is not statistically significant (p= 0.321), while the regression model that only uses uNGAL has less effect ($\chi 2^{=}$ 34.200 p < 0.001).

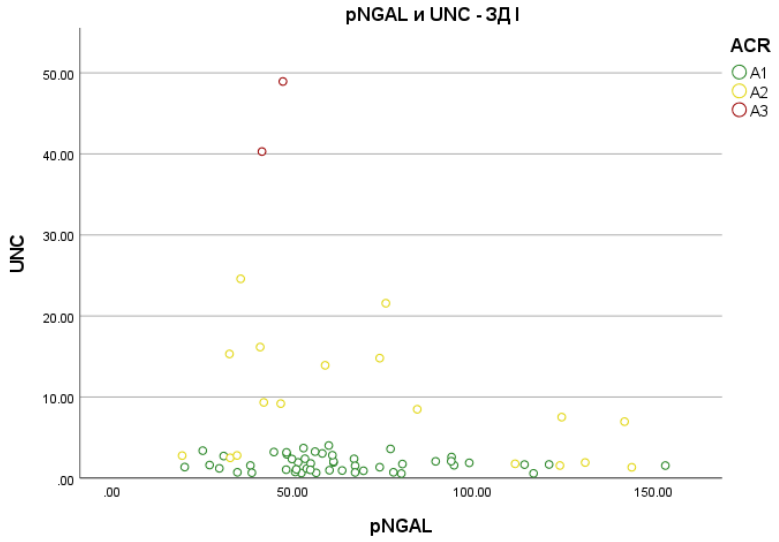


Figure 35. Distribution of pNGAL and UNC results in patients with DM I divided by ACR (DKD)

Three of the referred patients with DM I had significant leukocyturia and were excluded from the subsequent data processing ($\bar{x}$ - pNGAL=73.06 ng/ml, uNGAL= 128.60 ng/ml и UNC=12.23 µg/mmol) (Fig. 36).

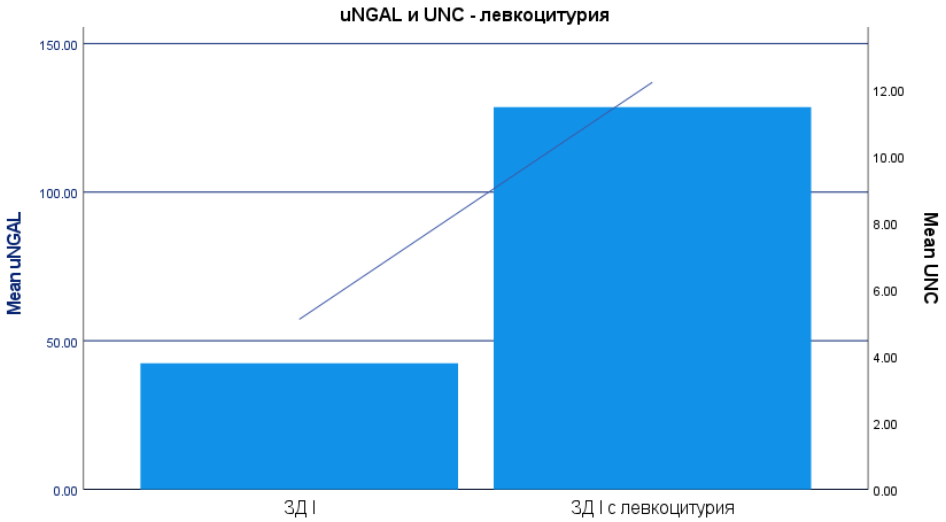


Figure 36. Mean – uNGAL and UNC in patients with DM I with and without leukocyturia

V. DISCUSSION

1. Verification of the immunoturbidimetric method for the determination of NGAL

The NGAL Test™ of the company Bioporto is a latex enhanced immunoturbidimetric analysis for the determination of NGAL with adaptations and applications for various analytical platforms. The NGAL measurement time of the ADVIA 1800 biochemical analyzer with which the study analysis was performed, was 10 min. NGAL Test™ is aimed at the diagnosis of acute renal injury (AKI) and has a measuring range of 25-3000 ng/ml, which enables the quantification of a larger range of values in this target group of patients. These characteristics determine the applicability of the test in clinical practice and in the diagnosis of ОБУ. The target groups in this study were healthy subjects and patients with DM with and without renal impairment. Data in the literature indicate that in some healthy subjects and in some patients with DM without DKD, a concentration of NGAL in urine at around and below the lower limit of quantification of NGAL Test™ can be expected. In order to optimize the test for the purposes of the study, the method was verified; the analytical measuring range was calculated and verified as well. Table 19 presents the analytical characteristics of NGAL Test™ for ADVIA 1800 analyzer, according to data from the company Bioporto, and it also presents the results obtained in the present study, and the acceptable criteria used in evaluating the analytical characteristics of the method. (Table 19)

Table 19. Analytical characteristics of NGAL Test™ on platform ADVIA 1800

Evaluated characteristic	Indicator	Bioporto	Study	Criteria
Detectable minimum	LLOD	12 ng/ml	6 ng/ml	3*SD-blank*
Linearity	LLOQ-ULOQ	25-3000 ng/ml	12-3000 ng/ml	<20% - <15%*
Irreproducibility in a series – Urine/Low	CV%	*	6.43%	<20%*
Irreproducibility in a series – Plasma/Low	CV%	9.6%	5.26%	<20%*
Irreproducibility in a series – plasma/clinically significant	CV%	*	1.85%	<20%*
Irreproducibility in a series – Plasma/High	CV%	*	1.82%	<20%*
Time Irreproducibility – Low Control	CV%	1.7%	4.5%	<20%*
Time Irreproducibility – High Control	CV%	0.9%	2.7%	<20%*
Unreliability with low control	d%	*	0.34	<10%, Westgard rules
Unreliability with low control	d%	*	0.40	<10%,Westgard rules
Analytical detectability - plasma	%recovery	*	98% (95:101)	80-120%**
Analytical detectability - urine	%recovery	*	98%	80-120%**
Transfer of biological material	%recovery	*	-0.29 %	<20% of LLOQ*

* EMEA/CHMP/EWP/2009 - Guideline on bioanalytical method validation (205)
** Practical Guide to Immunoassay Method Validation (206).

The verification of NGAL Test™ was carried out according to the guidelines of ISO 17025 – "Requirements for Method Verification" and the criteria for acceptability of the analytical characteristics are consistent with EMEA/CHMP/EWP/2009 "Guideline on bioanalytical method validation". The analytical reliability of the method was determined, while the influence of the biological material on the studied characteristics was also assessed. An LLOD value equal to 6.00 ng/ml and an LLOQ value equal to 12.00 ng/ml were established when assessing the measuring range. The resulting coefficients of variation upon LLOQ verification (x_0 =12.00 ng/ml - CV= 17.52%) and ULOQ (x_0 =3000.00 ng/ml – CV= 1.55%) are in accordance with international requirements and linearity of NGAL Test™ of ADVIA 1800 biochemical analyzer from 12.00 to 3000.00 ng/ml was applied for the purpose of the study. For the assessment of the random error, reproducibility in series and reproducibility in time was investigated. The data from the statistical processing showed very good reproducibility in a series with coefficients of variation in a low-reference, clinically relevant and pathological area, that were significantly lower than the acceptable criterion. The CV% derived from the reproducibility in time satisfy both the declared analytical characteristics (CV% < 5%) and the international requirements. In the unreliability assessment we found a low value of d% as a measure of systemic error and high analytical detectability as a measure of proportional systemic error. The data from carryover demonstrate that the analyzer provides very good sample separation without transferring biological material between them. When verifying the method of latex enhanced immunoturbidimetric analysis for determination of NGAL of the company Bioporto on the biochemical analyzer ADVIA 1800, very good analytical characteristics were found in accordance with the declared production and international standards. The test showed high analytical reliability in determining NGAL in both plasma and urine. However, the NGAL Test™ was unable to detect with the required accuracy any analyte concentrations of <12 ng/ml, as expected, in some healthy subjects. The results for reproducibility in series in the low area have a greater dispersion than in the reference and pathological area, which is independent of the biological material used. A higher CV% was found when assessing irreproducibility in time compared to the control material in the lower concentration area. Therefore, NGAL Test™ shows better analytical characteristics in the determination of high versus low analyte concentrations and is an appropriate test for the detection of pathological values that are expected in patients with renal impairment.

2. NGAL - reference interval. Biological variation

Currently, there are no international recommendations or guidelines available for NGAL analysis, there is insufficient information on biological variation, sources of analytical interference and optimal biological material for analysis. Most of the 'normal values' referenced in literature,

were measured in relatively large groups included in a clinical study as a control group. There were no large multicentre studies to derive NGAL reference limits stratified by age, gender and ethnicity.

In view of the lack of standardisation, the reference limits of pNGAL and uNGAL depend on the method, platform, biological material used for analysis, selection and distribution of the reference group. The UNC values between the different studies were further influenced by the analytical method for urinary creatinine and the measurement units used. In some of the cited studies UNC was presented in ng/mg (or µg/g), the conversion factor for recalculation of UNC from ng/mg to µg/mmol was 0.113 (1/8 .842). A comparative analysis evaluating reproducibility between several commercially available analyses for NGAL reported good comparability between results obtained from Bioporto ELISA and Bioporto PETIA and significant differences from other immunoassays. The authors point out as non-applicable the comparison of the results of different studies where there are different methods and analytical platforms used, and highlight the need to harmonise and standardise the analysis before this biomarker enters into routine practice.

2.1. NGAL - reference interval in adults. Biological variation

The appropriate material for the analysis of circulating NGAL levels is plasma. Serum is not recommended as falsely higher results may be reported due to neutrophil degranulation during clotting. A comparative analysis between NGAL levels in EDTA and heparin plasma showed higher values when using the anticoagulant lithium heparin, the authors recommend using identical biological material when comparing the results. The analytical manufacturer BioPorto reported expected values for pNGAL in EDTA plasma of 37-106 ng/ml (n=80). They indicate the following linear regression – NGAL(heparin) = 1.01xNGAL (EDTA) +16.6 (r^2=0.998) and specify that NGAL can be analysed from heparin plasma together with the other biochemical parameters, making it an appropriate indicator under emergency conditions. In the present study, heparin plasma was selected as material for analysis in determining circulating NGAL levels. This biological material is preferred as it allows simultaneous measurement of the concentration of pNGAL and that of standard and routine markers of renal impairment. The resulting reference limits will optimise the clinical relevance of NGAL Test^{TM} and the correct interpretation of the results for pNGAL.

Reference limits for NGAL in adults were determined in a cohort of 85 healthy subjects aged 34-75 years by immunoturbidimetric analysis. The defined reference interval of pNGAL in adults is 25–119.49 ng/ml, no significant difference in pNGAL values was found between males and females. Our results are comparable to the data in the literature in which pNGAL was measured with an identical reagent and methodology. Xiang D et al. (2013) determined NGAL levels in serum by particle-enhanced immunoturbidimetric analysis (PETIA) among 454 healthy volunteers aged 21-75 years. They indicate as upper limit of the reference range – 122.57 µg/l, based on the 95th

percentile. Makris K et al. (2015) determined the concentration of NGAL in EDTA plasma by PETIA among 200 healthy subjects aged 18-65 years. They reported sex-dependent pNGAL reference limits corresponding to the 5th and 95th percentiles in men – 38.7-157.6 ng/ml and in women – 24.4-142.5 ng/ml. In the study by I. Petrova et al. (2018), the defined control group of 16 subjects had baseline pNGAL values of 82.49 ±18.82 ng/ml as assessed in EDTA plasma by PETIA (Table 20).

The data processing in the present study found a positive correlation between pNGAL values and age and a negative correlation between eGFR and pNGAL. With increasing age, a significant decrease in eGFR was found to be consistent with an increase in pNGAL. The lowest mean eGFR value was reported in subjects >60 years, which is parallel to the higher pNGAL values in this age decade. Differentiation of the control cohort by age resulted in a reduction in the number of individuals in each group, which reduced the statistical significance of the data and limited the ability to derive age-dependent reference limits. Our positive correlation between pNGAL and age was cited in some of the studies. Xiang D et al., (2013) found a significant difference in pNGAL values between subjects below and above 45 years, and also defined an upper limit of the reference interval in the group below 45 years <116.52 µg/l and in the group above 45 years <126.9 µg/l. Makris K et al found a weak but significant correlation between pNGAL and age (rho= 0.177p=0.01). The observed trend can be explained by the physiological, age-related changes in glomerular filtration that cause an increase in circulating NGAL levels. With age, fibrosis of the intima of the interlobular arteries (arterial sclerosis) develops, which creates a hypoxic-ischaemic environment and leads to glomerulosclerosis and subsequent functional failure with a decrease in glomerular filtration. NGAL is a low molecular mass protein that is cleared from the circulation by renal excretion, undergoing glomerular filtration with subsequent tubular reabsorption. Therefore, any decrease in glomerular filtration reduces NGAL excretion with a consequent increase in systemic circulation. Table 20 shows the reference limits for pNGAL described in literature and encountered by us (Table 20).

Table 20. Reference limits for pNGAL in adults

Study	Count	Material	Method	Units	RL	RL-age	RL-sex (m/f)
BioPorto PI, 2011	80	EDTA-plasma	PETIA BioPorto	ng/ml	37-106	no	no
Xiang D, 2013	454	serum	PETIA BioPorto	µg/l	<122.6	<44 yrs <116.5 >45 yrs <126.9	no
Makris K, 2015	200	EDTA-plasma	PETIA BioPorto	ng/ml	28.7-167.0	no[1]	38.7–157.6/ 24.4–142.5
Petrova, I., 2018	16	EDTA-plasma	PETIA BioPorto	ng/ml	82.5±18.8	no	no
This study	85	heparin-plasma	PETIA BioPorto	ng/ml	25 – 119.5	no[1]	no

RL – reference limits

[1] no age-dependent reference limits were derived, but a significant positive correlation was found between pNGAL and age

Currently, there are no clear guidelines on whether normalization through the UNC ratio improves the diagnostic reliability of uNGAL as a tubular biomarker. Delanaye P et al. (2011) evaluated the biological intra-individual variation of uNGAL and UNC in samples from the first morning urine and random portions of urine and found a significant difference in uNGAL values (CV – 94%, 132%). They recommend using the UNC ratio to avoid daily variation in uNGAL values due to a difference in urine osmolarity. The authors emphasised the advantages of UNC compared to the absolute values of uNGAL under emergency conditions when using a random portion of urine. In the present study, first morning urine was selected as the analytical material for the determination of uNGAL levels. In view of the difficult collection of diuresis urine, a single portion of first morning urine is preferred, which is also standard material for routine urinalysis. The results for UNC are presented in μg/mmol.

Our upper limit of the uNGAL reference interval in first morning urine in adults was 52.37 ng/ml, no significant difference in uNGAL results was observed between the sexes. Data processing showed a statistically significant difference in UNC scores between males and females and sex-dependent reference values were defined for females < 6.34 μg/mmol and males < 3.36 μg/mmol.

Makris K et al. (2015) determined the concentration of uNGAL in a random portion of urine among 200 healthy subjects aged 18-65 years by PETIA and indicated, as the upper limit of the reference range, 54.5 ng/ml. Schinstock et al. (2013) measured uNGAL in diuresis urine by ELISA in 125 healthy volunteers aged 22-77 years. They found a statistically significant difference in uNGAL and UNC values between the two sexes and defined as the upper limit of the uNGAL reference range the following: in females, 65 ng/ml and males, 23.4 ng/ml; and UNC: in females, ≤89.9 ng/mg and males, ≤27.2 ng/mg. In the study of Zh. Hristova et al. (2015) are defined reference limits of uNGAL and UNC measured in first morning urine by ELISA method among 80 healthy subjects aged 21-47 years. They found a significant difference in values between the two sexes and defined sex-dependent reference values for uNGAL as follows: in females – 0.16-73.04 ng/ml and in males – 5.54-23.86 ng/ml, and for UNC in females – 0.08 – 42.8 ng/mg and in males – 5.24 – 20.72 ng/mg. The upper limit of the uNGAL reference range defined in the present study was comparable to the results of the Makris K et al. (2015) study using identical reagent and methodology. Close to our results are also the sex-differentiated reference limits of uNGAL and UNC by Schinstock et al. (2013) and Zh. Hristova et al. (2015), using the same manufacturer's ELISA method. Significantly higher reference values were measured in the study by Cullen MR et al. (2012) by chemiluminescence immunoassay uNGAL <107 μg/l and UNC <13 μg/mmol and in the study by Pennemans et al. (2013) in which the assay was performed by another manufacturer's ELISA test (2 to 5 times higher – uNGAL and UNC). Both studies confirmed a significant difference in uNGAL concentration between gender and age (Table 21, 22).

Table 21. Reference limits for uNGAL in adults

Study	Count	Material	Method	Units	RL	RL-age	RL-sex (m/f)
Makris K, 2015	200	spot urine	Petia BioPorto	ng/ml	< 54.5	no[1]	no
Schinstock C, 2013	125	diuresis urine	ELISA BioPorto	ng/ml	no	no	< 23.4/< 65.0
Hristova Zh., 2015	80	morning urine	ELISA BioPorto	ng/ml	<65.0	no	< 23.86/< 73.04
Cullen MR, 2012	174	spot urine	CLIA Abbott	µg/l	<107.0	19-40 yrs.< 91 40-59 yrs. <59 60-88 yrs. <250	<91.0 / <129
Pennemans V, 2013	338 (0-95 yrs.)	spot urine	ELISA Biovend.	µg/l	no	21-30 yrs.: <73.88/149.29 31-40 yrs: <87.54/153.60 41-50 yrs.: <103.95/158.37 51-60 yrs.: <123.70/163.62 61-70 yrs.: <146.52/169.38 71-80 yrs.: <176.31/175.68 >81 yrs.: <211.16/182.58	
This study	85	morning urine	PETIA BioPorto	ng/ml	<52.37	no	no

[1] a significant positive correlation was found between uNGAL and age

Table 22. Reference limits for UNC in adults

Study	Count	Material	Method uNGAL	Method uCreat	Units	RL	RL-age	RL-sex (m/f)
Schinstock C, 2013	125	diuresis urine	ELISA BioPorto	*	ng/mg	no	no	≤27.2/≤89.9
Hristova Zh., 2015	80	morning urine	ELISA BioPorto	enzymatic	ng/mg	no	no	<20.72/<42.8
Cullen MR, 2012	174	spot urine	CLIA Abbott	Jaffe-kinetic	µg/mmol	<13	19-40 yrs. <12 40-59 yrs. <8 60-88 yrs. <23	< 9/ <21
Pennemans V, 2013	338 (0-95 yrs.)	spot urine	ELISA Biovend.	Jaffe-kinetic	µg/g	no	21-30 yrs.: <125.5/243.2 31-40 yrs.: <122.1/236.5 41-50 yrs.: <127.6/247.0 51-60 yrs.: <142.9/276.6 61-70 yrs.: <171.4/332.2 71-80 yrs.: <221.0/428.9 >81 yrs.: <307.8/598.3	
This study	85	morning urine	PETIA BioPorto	Jaffe-kinetic	µg/mmol	no	no	< 3.36/ <6.34

RL – reference limits

In the present study, there was no statistically significant difference in uNGAL concentration between the two sexes, as cited in most literature sources. This could be explained by the influence of urine osmolarity on the absolute values of uNGAL when tested in a single portion of urine. After normalisation of uNGAL results to urinary creatinine results, the difference in UNC values between males and females reached great significance as described in the literature. This gender dependence in UNC values is most likely due to the influence of several factors that are emphasized. Experiments in mice have shown that oestrogens stimulate NGAL expression in a number of organs, such as the mammary gland and uterus. It is possible that oestrogen-mediated expression of NGAL in the renal

tubules may explain the difference in uNGAL values between men and women cited in some studies. Another possible explanation for higher UNC scores in women is the expected lower urinary creatinine concentrations due to lower muscle mass. In our study, there was a significant difference in urinary creatinine values between the two sexes, with higher results in men, which could explain gender dependence in UNC values.

Five of the subjects referred to the study had significant leukocyturia and were excluded from data processing. The mean value of uNGAL measured in these patients was 141.7 ng/ml and UNC – 23.32 μg/mmol. The results obtained for uNGAL and UNC were significantly higher than the defined reference range, which confirms the influence of leukocyturia and urinary tract infections on urinary LCN-2 concentration. NGAL is an acute phase protein whose concentration in the urine increases with urinary tract infections due to its release from activated neutrophils and its increased expression by damaged tubular cells.

2.2. NGAL - reference interval in children. Biological variation

Reference limits for NGAL in children were determined in a cohort of 42 healthy subjects aged 6-17 years, by immunoturbidimetric analysis. The biological material used for analysis in the determination of pNGAL is heparin plasma, while for the determination of uNGAL, the material is first morning urine. The derived reference limits in children are: for pNGAL < 96.88 ng/ml, for uNGAL < 47.30 ng/ml and for UNC < 3.48 μg/mmol. The data processing did not reveal a significant difference in sex or age for all three indicators.

The studies that established the NGAL reference range in children were not many, with the majority of the expected values reported in the literature in healthy children being derived from clinical trials with control groups evaluating the role of the marker in renal impairment. Van Donge T et al. (2020) measured pNGAL levels in EDTA plasma by PETIA among 142 healthy children aged 0-15 years. The authors set reference limits for pNGAL corresponding to the 5th and 95th percentiles, in children 1.4 - 78.6 μg/l, there was no significant difference in pNGAL values between different age groups, as between girls and boys. In the study by Papadopoulou-Marketou et al. (2017), the defined control group of 49 children aged 3-18 years had pNGAL values between 5.1-81.4 ng/ml (mean - 24.69±15.89 ng/ml) as measured by the ELISA method (Table 23).

Bennett MR et al. (2015) measured the levels of uNGAL in a random portion of urine, in a cohort of 368 healthy children 3-18 years of age, by ELISA and defined as the uNGAL upper limit of reference in children – 57.6 ng/ml. The study reported sex-dependent values for each age groups, as reported by Pennemans V et al. (2013). Rybi-Szumińska et al. (2013) measured the levels of uNGAL by ELISA in first morning urine in 172 children and derived UNC reference limits as follows: 0.2-5.9 yrs. - 33.91 ng/mg, 6-9.9 yrs. - 26.23 ng/mg, 10-13.9 yrs. - 20.29 ng/mg and 14-17.9 yrs. - 15.69

ng/mg (Table. 24, Tabl. 25).

Table 23. Reference limits for pNGAL in children

Study	Count	Age	Material	Method	Units	RL
Van Donge T, 2020	142	0.15	EDTA-plasma	PETIA BioPorto	ng/ml	1.4 – 78.6
Papadopoulou M, 2017	49	3-18	plasma:	ELISA BioPorto	ng/ml	5.1 - 81.4
Botsova V, 2018	15	7.8±1.8	*	ELISA	ng/ml	154.9 ± 35.4
This study	42	5-17 yrs.	heparin-plasma	PETIA BioPorto	ng/ml	<96.88

Table 24. Reference limits for uNGAL in children

Study	Count	Age	Material	Method	Units	RL – General Group (boys/girls)
Bennett MR, 2015	368	3-18 yrs.	spot urine	ELISA BioPorto	ng/ml	3-18 yrs.< 57.6 (28.3/73.1) 3-5 yrs.< 39.1 (26.1/52.2) 5-10 yrs.< 58.5 (10.9/139.5) 10-15 yrs.< 66.8 (25.5/72.3) 15-18 yrs.< 74.7 (50.0/138.6)
Cangemi G, 2013	308	0.63-248 months	spot urine	CLIA Architect	ng/ml	<58.7
Pennemans V, 2013	338 (0-95 yrs.)	0-20 yrs.	spot urine	ELISA Biovend	µg/l	0-10 yrs. < (52.97/141.80) 10-20 yrs. < (62.50/145.43)
Botsova V, 2018	15	7.8±1.8	*	ELISA	ng/ml	53.31±51.0
This study	42	5-17 yrs.	morning urine	PETIA BioPorto	ng/ml	<47.30

Table 25. UNC Reference limits for Children

Study	Count	Age	Material	Method NGAL	Method uCreat	Units	RL – General Group (boys/girls)
Rybi-Szumińska, 2013	172	0-18 yrs.	morning urine	ELISA R&D Systems	*	ng/mg	0.2-5.9 yrs. <33.91 6.0-9.9 yrs. < 26.23 10-13.9 yrs.< 20.29 14-17.9 yrs. < 15.69
Cangemi G, 2013	308	0.63-248 months	spot urine	CLIA Architect	*	ng/mg	UNC < 68.2
Pennemans V, 2013	338 (0-95 yrs.)	0-20 yrs.	spot urine	ELISA Biovend	Jaffe-kinetic	µg/g	0-10 yrs. < (167.3/325.4) 10-20 yrs.< (139.0/269.9)
Stoyanova L, 2018	30 (13 children)	23.6±12.8	morning urine	ELISA	Jaffe-kinetic	ng/mol	<0.190
This study	42	5-12 yrs.	morning urine	PETIA BioPorto	Jaffe-kinetic	µg/mmol	<3.48

The results obtained in our study were similar to those indicated in the literature, but not identical, which could be explained by the size and characteristics of the selected cohort of children and the method of analysis used. There was no gender or age statistically significant difference in pNGAL, uNGAL or UNC values, as cited in part of the studies. Age dependence in UNC values can be explained by changes in muscle mass that are typical as age increases, and are associated with higher

serum and urinary creatinine values. Our study found a negative correlation between UNC and age, which did not reach a statistically significant difference. It is necessary to include additional persons in different age groups in order to assess the physiological age-related changes of UNC in children and, if necessary, to derive reference limits differentiated by age.

The age- and sex- specific reference values of NGAL for the Bulgarian population are summarized in Table 26 (Table 26).

Table 26. The NGAL reference limits determined in this study

Indicator	Material	Method	Units	RL-men	RL-women	RL-children
UNC	1st morning urine	PETIA	µg/mmol	< 3.36	< 6.34	<3.48
uNGAL	1st morning urine	PETIA	ng/ml	<52.37		<47.30
pNGAL	hep. plasma	PETIA	ng/ml	25 – 119.5		<96.88

Data in the literature show that the comparison of results from studies using different methods and analytical platforms, is not applicable. In order to correctly interpret NGAL values, it is necessary to use gender, age and methodologically derived reference limits. The administration of normalized values of uNGAL (UNC) is recommended over the absolute concentration of the marker, when determined in a single portion, due to daily fluctuations in urine osmolarity. When interpreting UNC results, the influence of physiological changes in urinary creatinine values, the analytical method used to determine urinary creatinine, and the units of measure in which the ratio is presented should be taken into account. Assessment of uNGAL and UNC values should take into account urinary sediment results, taking into account the influence of leukocyturia and UTIs on the marker and ratio.

3. Diagnostic reliability of NGAL in the diagnostics of DM

DKD is a chronic complication of DM leading to increased cardiovascular morbidity and mortality and risk of developing ESRD. DN is taken as a disease primarily affecting the glomerulus leading to focal or diffuse glomerulosclerosis, however, a number of studies indicate the predictive role of tubulo-interstitial lesions in the progression of DN.

NGAL is one of the most promising tubular biomarkers in the diagnosis of kidney disease. Data in the literature indicate NGAL as a marker with a good diagnostic profile in the diagnosis of DKD. Studies conducted in patients with DM demonstrated that NGAL values correlated with AER progression, with the eGFR decline, with the severity of renal impairment, and with the risk of progression to ESRD. However, a number of causes outside the DKD may induce increased expression of NGAL. All this necessitates further research to clarify the role of NGAL in the diagnosis of DKD.

3.1.NGAL as a marker for the diagnosis of DKD in patients with DM II

According to the KDIGO 2020 guidelines, the criteria adopted in this study for the diagnosis of DKD are ACR >3 g/mol and/or eGFR <60 ml/min/1.73m². In order to assess the diagnostic reliability of NGAL for diagnosing DKD in patients with DM II, 92 patients with DM II were enrolled in the cohort, aged 18 to 89, of whom 55% were female and 45% were male. pNGAL was measured in heparin plasma and uNGAL in first morning urine, whereas the UNC results presented in µg/mmol. Patients were divided into groups depending on ACR, eGFR, HbA1c and the presence of DKD. The formula used to calculate the eGFR in patients with MD II is CKD-EPI.

Statistical data showed that pNGAL, uNGAL and UNC values across the cohort of patients with MD II were significantly higher than in the adult control group. Table 27 summarizes the medians of pNGAL, uNGAL and UNC in the control group and each of the subgroups of patients with DM II, divided by ACR, eGFR and depending on the presence of DKD (Table 27).

Table 27. Medians of pNGAL, uNGAL and UNC in the DM II cohort

Category	Criteria	N (%)	pNGAL (ng/ml) median (IQR)	uNGAL (ng/ml) median (IQR)	UNC (µg/mmol) median (IQR)
control group	clinically healthy	85	72.65 (33.38) [4,6,8]	14.50 (11.55) [3,4,5,6,8]	1.75 (1.25) [2,3,4,5,6,7,8]
DM II group	DM II	92	79.10 (64.98) [1]	32.30 (67.15) [1]	4.78 (9.05) [1]
A1	ACR <3 g/mol	41(46%)	71.10 (53.00)	19.75 (24.80)[3,4]	2.52 (2.49)[1,3,4]
A2	ACR – 3-30 g/mol	28(31%)	82.10 (50.87)	41.25 (61.85) [1,2]	7.86 (12.35)[1,2]
A3	ACR<30 g/mol	21(23%)	121.70 (104.55) [1]	91.30 (100.70)[1,2]	10.94 (25.63) [1,2]
≤G2	eGFR≥60ml/min/1.73m²	63(68%)	69.80 (41.00)[6]	28.45 (32.92)[1]	4.39 (10.51)[1]
≥G3	eGFR<60ml/min/1.73m²	29(32%)	121.90 (96.60)[1,5]	44.20 (57.06)[1]	6.21 (6.47)[1]
DM II without DKD	ACR<3 g/mol и eGFR≥60ml/min/1.73m²	36 (39%)	70.45 (40.38)	18.70 (21.90)[8]	2.26 (2.27)[1,8]
DM II with DKD	ACR>3 g/mol and/or eGFR<60ml/min/1.73m²	56 (61%)	93.55 (78.38)[1]	49.80 (77.40)[1,7]	8.54 (15.25)[1,7]

[1]significant difference to the control group, [2]significant difference to A1, [3]significant difference to A2, [4]significant difference to A3, [5]significant difference to ≤G2, [6]significant difference to ≥G3, [7]significant difference to DM without DKD, [8]significant difference to DM without DKD.

In the present study, pNGAL emerged as a marker for the assessment of glomerular filtration, whereas patients with DM II with reduced glomerular filtration, defined as eGFR<60 ml/min/1.73m², scoring statistically significantly higher for pNGAL than both control and patients with DM II with preserved glomerular filtration. The ROC analysis demonstrated good diagnostic

effectiveness of pNGAL in differentiating patients with DM II with eGFR <60 ml/min/1.73m² with AUC-ROC - 0.753. At cut-off 121.65 ng/ml, the marker has diagnostic effectiveness of 82% in distinguishing patients with DM II with reduced glomerular filtration. Significant correlation of pNGAL with markers for glomerular filtration assessment - positive with urea and creatinine concentration in plasma and negative with eGFR was found.

A majority of the studies found in the literature evaluated the role of pNGAL as a marker of DKD in patients with normal glomerular filtration. Bolignano D et al. (2009) found a positive correlation between pNGAL and serum creatinine and a negative correlation with GFR in a cohort of 59 patients with DM II with GFR>30 ml/min ($p<0.05$ and $p<0.01$). Kaul A et al. (2018) found a negative correlation between pNGAL and eGFR in a group of 144 patients with DM II with eGFR>60 ml/min/1.73m^2. Żyłka A et al (2018) evaluated the predictive value of glomerular and tubular markers as indicators of early renal impairment in 80 patients with DM II with a median eGFR=92.4 ml/min/1.73m^2. The study retrospectively monitored changes in eGFR and their relationship with the biomarkers tested, the results show a decrease in glomerular filtration in 17 patients. The authors indicate that a pNGAL value above 61 μg/l was significantly associated with a decrease in eGFR.

GFR is a laboratory marker evaluating renal function and is also a CKD classification criterion. The gold standard in the determination of GFR is a measurement of the clearance of the exogenous substance inulin. This method is invasive, requires a lot of work, and is not used in routine practice. Currently, in order to assess GFRs in adults, creatinine-based formulas such as MDRD and CKD-EPI are used, which include variables depending on age, gender and ethnicity. They are based on a serum creatinine concentration determined by a method whose calibration is traceable to a reference method ID-MS. MDRD and CKD-EPI have a P30 value between 80% and 90%, which means that the eGFR generated by these formulas has a 90% chance of being within ± 30% of the real GFR. CKD-EPI demonstrated significantly greater accuracy than MDRD when calculating eGFR in the subgroup with GFR > 90 ml/min/1.73 m². Therefore, CKD-EPI is preferred in patients with DM since the earliest haemodynamic abnormality is the glomerular hyperfiltration with an increase in GFR >120 ml/min/1.73m².

The relationship between glomerular filtration and pNGAL concentration observed by us and in part of the studies can be explained by the renal protein excretion, whereas any decrease in glomerular filtration decreases the clearance of NGAL with its subsequent accumulation in the systemic circulation. Characteristic morphological changes in DN include thickening of the glomerular basal membrane, mesangial expansion, hyalization of the afferent and efferent arterioles, reduction of the numerical density of podocytes, nodular or diffuse glomeruloscerosis, etc. These structural changes resulted in subsequent functional failure, impaired renal excretory function and a

decrease in glomerular filtration.

In the present study, there was no correlation between pNGAL and ACR. Only patients in A3 had statistically significantly higher scores compared to the control group, which was consistent with the expected more severe renal impairment and more reduced glomerular filtration. A number of studies have indicated the clinical relevance of pNGAL in the diagnosis of pathological albuminuria and renal impairment in patients with DM II. Bolignano D et al. (2009) found significantly higher pNGAL scores in patients with DM II with normal albumin excretion compared to controls (52.5 vs 233.6 ng/ml), but found no significant correlation between pNGAL and ACR. Mahfouz MH et al. (2016) reported higher pNGAL values in patients with DM II with moderately and strongly elevated ACR relative to the reference group (97.8, 131 vs 46.5 ng/ml) and indicated a positive correlation of the indicator with ACR (r= 0.582, p=0.0001). A meta-analysis evaluating the role of pNGAL in the diagnosis of DKD found a combined sensitivity and specificity - 79% and 87%, a combined ratio of the positive (PLR) and negative probability – 5.97 and 0.24. The analysis summarises that pNGAL is an appropriate marker for the confirmation of DKD.

The mean and cut-off NGAL values for DKD found in the literature differ significantly from each other, which can be explained by the heterogeneous conditions of analysis. Which confirms the thesis that a comparison of NGAL results between different studies is not applicable and indicates the need for harmonisation and standardisation of the analysis (Table. 28).

Table 28. Mean values/median pNGAL in patients with DM II

Study	CG	A1	A2	A3	Units	+ ACR p<0.05	- GFR p<0.05	Method
Bolignano D, 2009	52.5	233.6[3]	290.4[3]	890.9	ng/ml	No	Yes	ELISA Gentofte
Mahfouz MH, 2016	46.4[1]	55.6[1]	97.8	131.0	ng/ml	Yes	Yes	ELISA R&D
Motawi TK, 2017	45.5[1]	55.7[1]	97.9		ng/ml	No	No	ELISA R&D
Wu C, 2014	59.0	138.1	245.2[4]	255.5[4]	ng/ml	Yes	-	ELISA EIAab&U
Siddiqi Z, 2017		126.7	155.1		ng/ml	Yes	-	ELISA R&D
Żyłka A, 2018		53.8	67.2		µg/l	No	No	ELISA BioVendor
Kaul A, 2018	42.6	113.6	263.2	474.9	ng/ml	Yes	Yes	ELISA R&D
Forghani MS, 2020		70.0	99.8		ng/ml	Yes	No	ELISA Biotech.
This study	72.6[1]	71.1[1,3]	82.1[2,3]	121.7	ng/ml	No	Yes	PETIA Bioporto

[1] no significant difference between A1 and the control group (p>0.05), [2] no significant difference between A2 and the control group (p>0.05), [2]no significant difference between A2 and A1 (p>0.05), [3] no significant difference between A3 and A2 (p>0.05)

Abbreviations: CG– control group; A1-patients with DM with normal AER/ACR; A2- patients with DM with moderately increased AER/ACR; A3- patients with DM with strongly increased AER/ACR; +ACR –significant positive correlation with AER/ACR (p<0.05); -GFR - significant negative correlation with GFR (p<0.05).

In the natural course of renal impairment in DM, the decrease in eGFR is most often a late indicator of the presence of DKD. Therefore, biomarkers that correlate only with the decrease in eGFR but not with albuminuria are not suitable for diagnosis of DKD. This finding can also explain the results we have obtained and the conclusion that the use of pNGAL alone does not have the necessary effectiveness for routine diagnosis of DKD in patients with DM II.

The present study found that in the cohort of patients with DM II, the values of uNGAL and UNC correlated significantly with changes in albumin excretion, with UNC showing a better dependence with ACR than uNGAL. The values of uNGAL and UNC increase progressively from A1 to A3, whereas a significant difference is found between A1 vs A2 and between A1 vs A3. Patients with DM II with moderately and strongly elevated ACR scored statistically significantly higher for uNGAL and UNC than the control group, and patients with normoalbuminuria scored significantly higher for UNC. Sex difference in UNC values between subgroups of patients divided by ACR and eGFR was outlined. Women scored higher for UNC than men in each of the subgroups, with a significant difference only in subgroup A1 and subgroup eGFR $\geq$ 60 ml/min/1.73m^2. The difference is decreasing with the progression of renal impairment. Therefore, sex-dependent cut-off UNC values for renal impairment were derived.

The ROC analysis demonstrated good diagnostic effectiveness of uNGAL in differentiating patients with DM II with elevated ACR (A1 vs A2/A3) with AUC-ROC – 0.776. The diagnostic effectiveness with which a value of uNGAL >53.30 ng/ml correctly identifies a patient with DM II with ACR >3.0 g/mol, is 67%. The UNC ratio showed very good diagnostic accuracy in distinguishing patients with DM II with albuminuria (A1 vs A2/A3) with AUC-ROC in females – 0.845 and in males – 0.904. At cut-off value in women of 6.87 µg/mmol, and in men – 3.72 µg/mmol, the diagnostic effectiveness of the marker was 80% and 92% in detecting patients with DM II with ACR >3.0 g/mol.

The values of uNGAL and UNC were significantly higher in patients with DM II with DKD (defined as ACR >3 g/mol and/or eGFR<60 ml/min/1.73m^2) compared to patients with DM II without DKD and the control group. Patients with DM II without DKD also had statistically significantly higher UNC scores than the reference group.

The ROC analysis demonstrated good diagnostic reliability of uNGAL (AUC-ROC – 0.787) and UNC (AUC-ROC in females – 0.845 and in males – 0.904) in distinguishing the patients with DKD among the subjects with DM II (DM without DKD vs DM with DKD). The diagnostic effectiveness with which a value of uNGAL >53.30 ng/ml is able to correctly identify patients with DM II with DKD is 65%. At the cut-off value of UNC in females of 6.87 µg/mmol and in males of 3.72 µg/mmol, the marker has diagnostic effectiveness of 69% and 89% in distinguishing patients with DM II with DKD. UNC has better diagnostic accuracy than uNGAL in diagnosing patients with

DKD. The use of this marker alone statistically significantly differentiates patients with DM II with renal impairment. The increase in UNC by 1 μg/mmol in patients with DM II increases the likelihood of DKD in females 1.16-fold and in males 2.40-fold.

Most of the studies in patients with DM II, which are found in the literature, defined DKD as an increase in AER/ACR. Bolignano D et al. (2009) found in patients with DM II a positive correlation between uNGAL and ACR ($p<0.01$) and a negative correlation with GFR ($p<0.005$), a significant difference in uNGAL values between the patient groups separated by stopped ACR and significantly higher scores compared to the control group (6.5 vs 51.1 vs 105.9 vs 255.6 ng/ml). Żyłka A et al. (2018) investigated the role of glomerular and tubular markers in the diagnosis of DKD in patients with DM II and found a significant correlation between ACR with uNGAL ($p<0.001$) and with UNC ($p=0.002$), as well as significantly higher values of uNGAL and UNC in the group with ACR >30 mg/g compared to the cohort with ACR < 30 mg/g (24.7 vs 10.3 μg/l, 35.1 vs 9.02 μg/g). Data from other studies confirmed the clinical relevance of uNGAL and UNC in the diagnosis of renal impairment among patients with DM II. Literary sources indicate that the results of uNGAL and UNC correlate with the progression of albumin excretion, the decline in eGFR and the severity of DKD in patients with DM II (Table 29, 30).

Table 29. Mean values/medians of uNGAL in DM II

Study	Indicator	CG	A1	A2	A3	Units	+ ACR p<0.05	- GFR p<0.05	Method
Bolignano D, 2009	uNGAL	6.5	51.1	105.9	255.6	ng/ml	Yes	Yes	ELISA Gentofte
Siddiqi Z, 2017	uNGAL		4.8	10.3.		ng/ml	Yes	-	ELISA R&D
Żyłka A, 2018	uNGAL		10.3.	24.7		μg/l	Yes	No	CLIA Abbott
Assal HS, 2013	uNGAL	4.3	7.4	10.1.	17.4.	ng/ml	Yes	-	ELISA BioVendor
Sueud T, 2019	uNGAL	40.5	58.7	64.0	72.8	ng/ml	No	-	ELISA Elabscience
Abbasi F, 2020	uNGAL	77.0	114.1	122.1[3]	129.1[3]	ng/mg	-	Yes	ELISA BioassayT.
Vijay S, 2018	uNGAL	26.6	146.1	228.2		ng/ml	Yes	-	ELISA -
Kaul A, 2018	uNGAL	7.9	31.3	110.3	352.9	ng/ml	Yes	Yes	ELISA R&D
Forghani MS 2020	uNGAL		70.0	99.8		ng/ml	Yes	No	ELISA Biotech.
Current Survey	uNGAL	14.5[1]	19.7[1]	41.2[3]	91.3[3]	ng/ml	Yes	Yes	PETIA Bioporto

[1] there is no significant difference between A1 and control group ($p>0.05$)
[3] there is no significant difference between A3 and A2 ($p>0.05$)
Abbreviations: CG– control group; A1-patients with DM with normal AER/ACR; A2- patients with DM with moderately increased AER/ACR; A3- patients with DM with strongly increased AER/ACR; +ACR –significant positive correlation with AER/ACR ($p<0.05$); -GFR - significant negative correlation with GFR ($p<0.05$).

Table 30. Mean values/medians of UNC in DM II

Study	Indicator	CG	A1	A2	A3	Units	+ ACR p<0.05	- GFR p<0.05	Method
Żyłka A, 2018	UNC		9.02	35.1		µg/g	Yes	No	CLIA Abbott
Fu WJ, 2012	UNC	32.9	69.2	123.6	235.0	µg/g	Yes	Yes	ELISA R&D
Kaul A, 2018	UNC	7.0	31.3	98.2	273.2	ng/g	Yes	Yes	ELISA R&D
*Fufaa GD, 2014	UNC		5.39	6.92	9.81	ng/mmol	Yes	No	CLIA Abbott
*Current study	UNC	1.75	2.52	7.86	10.94	µg/mmol	Yes	Yes	PETIA Bioporto

* statistically significant difference in UNC values between men and women was found

Meta analysis evaluating the role of uNGAL and UNC in the diagnosis of DKD found that in differentiating patients with DM II with pathological albuminuria, uNGAL had a combined sensitivity and specificity of 83% and 81% with AUC-ROC – 0.89, and UNC had a combined sensitivity and specificity of 42% and 73% with AUC-ROC - 0.69. The analysis found that uNGAL and UNC are valuable biomarkers for the diagnosis of DKD in patients with DM II, and their diagnostic accuracy varies from moderate to high according to AUC-ROC. Tang XY et al. (2019) summarize data on the role of uNGAL in distinguishing patients with ACR >3 g/mol among patients with DM II and indicate a combined sensitivity and specificity of 82% and 81% with AUC-ROC-0.88 in cross studies and 96% and 89% with AUC-ROC-0.98 in cohort studies. The authors found that the accumulated evidence showed the efficacy of uNGAL as a marker of renal impairment in DM (Table 31).

Table 31. Diagnostic effectiveness of uNGAL and UNC in distinguishing patients with DM II with ACR >3.0 g/mol

Study	Indicator	Cut-off	Units	DSen	DSpec	LR+	LR-	PPV	NPV	DE
Sueud T, 2019	uNGAL	21.4	ng/ml	94%	26%	1.28	0.20	50%	15%	16%
Żyłka A, 2018	uNGAL	14.3	µg/l	80%	61%	2.05	0.32	39%	90%	65%
Kaul A, 2018	uNGAL	49.0	ng/ml	90%	83%	5.29	0.12	90%	83%	87%
Assal HS, 2013	uNGAL	8.8	ng/ml	67%	89%	6.09	0.37	89%	69%	77%
current study	uNGAL	53.30	ng/ml	47%	90%	4.67	0.59	84%	60%	67%
Żyłka A, 2018	UNC	28.3	µg/g	60%	87%	4.61	0.46			
Kaul A, 2018	UNC	50.0	ng/g	95%	89%	8.63	0.06	93%	91%	93%
Fufaa GD, 2014	UNC	8.0	ng/mmol	25%	81%	1.32	0.92	41%	67%	62%
current study	UNC–women	6.87	µg/mmol	75%	86%	5.24	0.29	88%	72%	80%
current study	UNC – men	3.72	µg/mmol	88%	95%	16.64	0.12	94%	90%	92%

DSen – diagnostic sensitivity, DSpec – diagnostic specificity, LR+ – positive probability ratio, LR- – negative probability ratio, PPV – positive predictive value, NPV – negative predictive value, DE – diagnostic effectiveness

Currently, AER/ACR is the earliest laboratory marker for the presence of DKD, a criterion for classification of DKD and a predictor of cardiovascular complications in DM. The association between uNGAL and UNC with AER/ACR observed in patients with DM II may be due to common and potentiating mechanisms in DKD that lead to pathological excretion of both proteins. Albumin is a negatively charged protein with a molecular mass of 69 kDa that appears in the urine at a pathological concentration in case of loss of the 'loading sieve' of the glomerular filtration barrier and is the best marker for selective glomerular proteinuria. The development of DN results in damage to the glomerular filtration barrier including capillary endothelium, glomerular basal membrane and podocytes, which causes increased excretion of albumin. However, there is increasing evidence that tubular damage also plays a role in the development of pathological albuminuria, which limits its reabsorption by the megaline/cubiline multiligand receptor. Animal studies have shown that in the early stages of DKD, albumin reabsorption decreases without increasing its glomerular filtration, which can be explained by reduced albumin endocytosis or reduced expression of the megaline/cubiline receptor. According to the "extraction hypothesis", the glomeruli filter high levels of albumin, which appears in the urine in a pathological amount only if a disturbance in the tubular reabsorption occurs. On the other hand, pathological "glomerular" albuminuria may exceed the capacity for reabsorption of the damaged tubular cells. Persistent proteinuria is also a cause of progressive damage to the renal parenchyma, which can accelerate the development of DKD by activating inflammatory pathways in proximal epithelial cells and initiating tubulointerstitial inflammation and fibrosis. Pathological albuminuria may therefore also be an indicator of tubular damage. NGAL is a positively charged protein (pI >7.4) of low molecular mass (25-70 kDa) which is freely filtered through the glomerulus but is reabsorbed into the proximal renal tubules by megaline/cubiline-dependent endocytosis. Via In situ hybridisation it has been proven that in response to tubular damage, there is an intensive expression of NGAL mRNA from the Henle's loop and the collecting tubes. Therefore, increased NGAL excretion may occur in renal injury that affects its reabsorption in the proximal tubule and/or induces its de novo synthesis in the distal tubule. This explains why NGAL was designated as a tubular marker. Proteins NGAL and albumin have a common mechanism of reabsorption in the proximal tubules and at any renal tubal damage, increased excretion of both proteins can be expected. At the same time, the pathomorphological changes in DN affect both the glomerulus and the tubulointerstitium of the kidney, with abnormal proteinuria also playing an important role in the pathogenesis of tubular damage, therefore an increase in both glomerular (albumin) and tubular biomarkers (albumin and NGAL) can be expected in DKD.

In our study it was found that patients with DM II with ACR<3 g/mol and patients without renal impairment had higher UNC scores compared to the adult control group. The results of a number of

studies demonstrated significantly higher plasma and urine concentrations of NGAL in patients with DM with normal albumin excretion rates compared to the control group. Kaul A et al. (2018) investigated the levels of UNC in patients with DM II and found a significant difference in the values of the indicator, both between the individual stages of DKD and between patients with DM II with normal ACR and controls. They determined the cut-off value of UNC 24.96 ng/mg in the identification of DKD with normal ACR with AUC-ROC – 0.996. The authors described NGAL as a marker of tubulointerstitial impairment that can detect the development of DKD prior to its diagnosis with the elevated AER, and identified NGAL as a useful and non-invasive indicator for the diagnosis of DKD.

Elevated AER is often the first clinical indicator of the presence of DKD, but is not always a marker of renal dysfunction in diabetes. DKD may also occur in patients with normal AER in whom there is a non-albuminuric phenotype of renal impairment. Also, not all patients with pathological albuminuria develop progressive renal dysfunction. Histological changes in the tubulointerstitium that can be observed in DN are tubulo-epithelial cell hypertrophy, tubular basal membrane thickening, epithelial-mesenchymal transition, glycogen accumulation, tubulointerstitial fibrosis and tubular atrophy. Some of these changes develop primarily and are not induced by glomerular pathology. Duan S et al. (2021) point and emphasise the role of structural and functional changes in renal tubules that can initiate and stimulate renal dysfunction in DM, and summarise that there is enough evidence accumulated to suggest a change in the traditional paradigm for the development of DN – from glomerulocentric to tubulocentric. According to the hypothesis of "tubular phase" of DKD, the tubular damage is a primary disorder initiating the development of renal dysfunction in patients with DM, therefore a structural tubular marker such as NGAL may be an earlier laboratory sign of DKD than AER.

The pathogenesis of DN is complex and multifactorial, and in its development involves hemodynamic and metabolic factors induced by hyperglycaemia and/or insulin deficiency such as hypoxia, oxidative stress, activation of a number of inflammatory and apoptotic pathways, etc. Multiple structural and functional changes can occur simultaneously and progress at different rates in the diabetic kidney, resulting in high heterogeneity of the disease. Although the exact chronological sequence of events is not well defined, both pathognomonic morphological changes in the glomerulus (glomerulosclerosis) and tubulointerstitial lesions are the cause of the clinical manifestations of DKD. In view of this, in order to diagnose early any DKD, biomarkers with high specificity to different aspects of renal impairment in DM II are needed.

In the natural course of renal impairment in MD II, ACR elevation is most often the first clinical indicator of the presence of DKD. Biomarkers that correlate with albuminuria are therefore suitable for the detection of DKD. This finding can also explain the results we have obtained that uNGAL

and UNC have the necessary diagnostic reliability in distinguishing the patients with DKD among the persons with DM II (DM without DKD vs DM with DKD). UNC showed better diagnostic efficiency than uNGAL in diagnosing patients with DKD. The use of this marker alone statistically significantly differentiates patients with DM II with renal impairment. (Table 32).

Table 32. NGAL - Cut-off values for DKD – ACR >3 g/mol and/or eGFR<60ml/min/1.73m²

Indicator	Cut-off	Units	DSen	DSpec	LR+	LR-	PPV	NPV	DE
uNGAL	53.3	ng/ml	45%	94%	7.64	0.58	92%	53%	65%
UNC – women	6.87	µg/mmol	64%	81%	3.38	0.44	88%	52%	69%
UNC – men	3.72	µg/mmol	83%	94%	14.88	0.18	94%	85%	89%

The gender dependence found by us in the results of the UNC is also quoted by Fufaa GD et al. (2014) – they found a median in females 9.4 ng/mmol and in males 1.8 ng/mmol among patients with DM II. This gender differentiation in UNC values may be due to oestrogen-mediated expression of NGAL in renal tubules and/or expected lower urinary creatinine concentrations in women as a result of lower muscle mass. In our study, the cohort of men had higher urine creatinine scores than the cohort of women with DM II, which could explain higher UNC scores in women compared to men with DM II. However, the difference between the two sexes proved to be significant only in patients with DM II with normal renal function and decreased in parallel with the progression of renal impairment. As DKD develops and progresses, tubular damage that causes reduced reabsorption or increased expression of NGAL becomes a determining factor for its concentration in urine and for the resulting UNC values. All this reduces the importance of gender differences in uNGAL and uCreatinine concentrations and their impact on UNC outcomes.

3.2. NGAL as a marker for DKD progression in patients with DM II

According to the KDIGO 2020 guidelines, patients with DM II are classified according to severity and prognosis of DKD compared to the values of ACR and eGFR in 4 groups (109). Depending on the glycaemic control, two subgroups of patients were formed. Since poor glycaemic control is a major factor in the development and progression of DKD, its relationship to the NGAL levels in plasma and urine has been evaluated. Table 33 presents the median and interquartile ranges of pNGAL, uNGAL and UNC in the each of the subgroups (Table 33).

The values of pNGAL, uNGAL and UNC progressively increased with the degree of renal impairment, but this difference only reached significance in the results of uNGAL and UNC. The values of uNGAL and UNC in the low-risk group differ statistically significantly with their respective scores in all other groups divided by severity and prognosis of DKD (moderate, high and very high risk). There was no significant difference in pNGAL, uNGAL and UNC values between

groups with good and poor glycaemic control.

Table 33. Median pNGAL, uNGAL and UNC in each of the subgroups of patients with DM II

Category	N (% of group)	pNGAL (ng/ml) median (IQR)	uNGAL (ng/ml) median (IQR)	UNC (µg/mmol) median (IQR)
DM II (whole cohort)	92	79.10 (64.98)	32.30 (67.15)	4.78 (9.05)
low risk	36 (39.1%)	70.45 (40.38)	18.70 (21.90)[2,3,4]	2.26 (2.27)[2,3,4]
moderate risk	20 (21.7%)	75.55 (41.05)	36.50 (37.95)[1]	7.85 (13.53)[1]
high risk	21 (22.8%)	114.65 (103.18)	47.00 (86.93) [1]	6.21 (30.18)[1]
very high risk	15 (16.3%)	121.80 (116.35)	99.80 (105.00)[1]	9.77 (10.87)[1]
good control	20 (27%)	87.60 (106.38)	27.10 (30.70)	3.98 (8.32)
poor control	54 (73%)	73.80 (67.95)	32.40 (44.08)	4.86(11.45)

[1]significant difference with the low-risk group, [2]significant difference with the moderate-risk group, [3]significant difference with the high-risk group, [4]significant difference with the very high-risk group

There was a statistically significant correlation between pNGAL and markers for glomerular filtration assessment – positive correlation with urea and creatinine and negative with eGFR. uNGAL showed a significant correlation with AER and ACR and negative with eGFR. UNC correlated statistically significantly positive with AER, ACR and HbA1c and negative with eGFR. No significant correlation was found for all three markers with metabolic control assessment parameters.

The simultaneous use of the two markers, pNGAL and UNC, demonstrates a more accurate assessment of the prognosis of DKD than their application alone, whereas both markers are statistically significantly related to the distribution of patients by group, relative to the severity of DKD. The model using pNGAL and UNC is able to statistically significantly discriminate between DKD groups and successfully predicts the distribution of 57% of patients with DM II in the respective groups, relative to the degree of renal impairment and their prognosis. The combined application of the two markers – pNGAL and UNC successfully predicts the presence of DKD with high and very high risk among patients with DM II. The increase in pNGAL by 1ng/ml increases the chance that a patient with DM II will have a DKD with high and very high risk by 1.01 times, and the increase in UNC by 1 µg/mmol increases the probability that a patient with DM II will have a DKD with high and very high risk by 1.09 times (women – 1.07 times, men – 1.14 times).

Data in the literature indicate the predictive role of tubulo-interstitial lesions in DN progression. Okada T. et al. (2012) noted that interstitial inflammation and fibrosis and tubular atrophy in DN patients are major factors associated with renal prognosis and development of ESRD. Mise K et al. (2015) demonstrated the importance of IFTA-score for assessing the risk of progression of renal

impairment in patients with DM. Hoshino J et al. (2018) developed a risk stratification system in DN patients based on the characteristic pathomorphological changes, whereas that system included both glomerular and tubolointerstitial lesions. This outlines the role of tubular markers as determinants of early decline of renal function in patients with DM. A number of clinical studies in a different model evaluated the role of NGAL as a marker of DKD progression. Hwang S et al. (2017) investigated the relationship between tissue expression of NGAL and KIM-1, as well as histopathological and laboratory parameters for renal progression in patients with proven DN. The study found that IFTA-score positively correlated with a decline in eGFR and found that tubulointerstitial damage was the most important histological finding in DN. They found that tissue expression of NGAL and KIM-1 correlated negatively with eGFR, but only the tissue expression of NGAL was an independent predictor of decline in eGFR. Satirapoj B et al. (2016) found that patients with DM II with higher levels of uNGAL had a faster decline in renal function, UNC > 772 ng/g predicted a decline in eGFR>25% per year, with AUC-ROC – 0.64. They highlight the crucial role that tubular damage plays in the progression of DKD and its development to the terminal stage. Nowak N et al. (2018) evaluated baseline levels of glomerular and tubular markers associated with early decline in renal function, following a large cohort of patients with DM II with normal eGFR over a period of 5-12 years. They found that UNC was significantly higher in the group with a rapid decline in renal function, defined as a decrease in eGFR >30% over 5 years, compared to patients with a lower decline in eGFR.

A number of studies also evaluated the relationship between poor control of DM and the development of LM with NGAL levels. Al-Refai et al. (2014) found in a cohort of patients with DM II a positive correlation between logUNC and HbA1c, regression analysis indicated HbA1c as an independent predictor of logUNC ratio. Mahfouz MH et al. (2016) found a significant correlation between pNGAL and the duration of diabetes, systolic and diastolic blood pressure, HbA1c, HOMA-IR and triglycerides in patients with DM II.

In the recommendations of KDIGO 2020, eGFR and AER or ACR are the main criteria for stratification of DKD. Therefore, the use of one or more markers that correlate with changes in both indicators will achieve a better assessment of the severity and prognosis of DKD. The results of our study are consistent with this finding. The data show that pNGAL correlates with the decrease in glomerular filtration, while UNC correlates with ACR. The simultaneous use of the results for pNGAL and UNC significantly predicts the severity of DKD, whereas pNGAL significantly improves the prognostic value of UNC in the stratification of DKD among patients with DM II.

Diabetes Control and Complications Trial Research Group indicate that long-term cumulative glycaemic exposure is a major risk factor for the development of DKD. Higher triglyceride levels and systolic blood pressure are also independent determinants of renal impairment in patients with

DM. Consistent with these data, our study found a significant correlation between UNC and HbA1c, most likely an expression of more severe renal impairment in patients with poor glycaemic control. In our cohort, there was no significant correlation between pNGAL, uNGAL and UNC with laboratory markers evaluating metabolic control.

3.3. NGAL as a marker for the diagnosis and prognosis of DKD in patients with DM I

In order to assess the diagnostic reliability of NGAL as a marker for DKD in patients with DM I, 75 children aged 6 to 17 years were included in the cohort, with a duration of DM I - 7.09 ±2.59 years, of which 44% were girls and 56% were boys. pNGAL was measured in heparin plasma and uNGAL – in first morning urine. The results for UNC are presented in µg/mmol. Patients were divided into groups depending on ACR, eGFR, HbA1c values. In this patient cohort, renal impairment was found in a small number of children with DM I, which is consistent with the relatively recent onset of DM I.

Statistical data showed that only UNC scores were significantly higher in the overall patient cohort of individuals with DM I, compared to the control group of children. Table 34 summarizes the medians and interquartile ranges of pNGAL, uNGAL and UNC in individual subgroups of patients with DM I divided by ACR, eGFR and HbA1c values (Table 34).

Table 34. Median and IQR of pNGAL, uNGAL and UNC in subgroups of patients with DM I

Category	N (% of the group)	pNGAL (ng/ml) median (IQR)	uNGAL (ng/ml) median (IQR)	UNC (µg/mmol) median (IQR)
control group	42	66.15(28.45)	13.70(10.35)	1.47(1.06)
DM I (whole cohort)	75	58.20(33.40)	13.00(15.00)	1.94 (2.14)
A1 (ACR<3 g/mol)	53 (72%)	57.75(27.85)	12.00(7.57)[3]	1.64(1.56)[3]
A2 (ACR – 3-30 g/mol)	19 (25%)	66.50(88.78)	41.4(66.10)[1,2]	8.49(12.82)[1,2]
$eGFR_{(Bedside\ Schwartz)}$ ≥90	15 (21%)	56.50(27.95)	12.35(17.25)	2.00(2.22)
$eGFR_{(Bedside\ Schwartz)}$<90	55 (79%)	80.60(80.00)	21.30(15.93)	2.00(7.20)
$eGFR_{(CKD\text{-}EPI40)}$ ≥90	24 (32%)	53.95(25.67)[4]	14.28(23.32)	2.37(3.83)
$eGFR_{(CKD\text{-}EPI40)}$ <90	50 (68%)	77.10(57.70)[5]	12.00(10.95)	1.90(1.29)
good control	33 (45%)	59.00(43.2)	12.00(8.90)	1.71(1.40)[7]
poor control	41 (55%)	54.90(35.6)	16.10(27.90)	2.79(6.70)[1,6]

[1]significant difference with control group, [2]significant difference with A1, [3]significant difference with A2, [4]significant difference with $eGFR_{(CKD\text{-}EPI40)}$ < 90, [5]significant difference with $eGFR_{(CKD\text{-}EPI40)}$ ≥ 90, [6]significant difference with good glycaemic control, [7] significant difference with poor glycaemic control

eGFR in patients with DM I was calculated using the established formula of 'Bedside Schwartz' – $GFR_{(Bedside\ Schwartz)}$ and the alternative formula CKD-EPI40 - $eGFR_{(CKD\text{-}EPI40)}$. It is preferred to apply and evaluate both because the Bedside Schwartz formula has been validated in children with reduced glomerular filtration. The regression analysis we performed found a very good relation between the two formulas used and a lack of statistically significant difference when comparing the results.

The present study found a negative correlation between pNGAL and the two formulas used to calculate eGFR, but it was only significant with $eGFR_{(CKD\text{-}EPI40)}$. Results for pNGAL were found to be statistically significantly lower in patients with DM I with $eGFR_{(CKD\text{-}EPI40)} \geq 90$ ml/min/1.73 m² compared to those with $eGFR_{(CKD\text{-}EPI40)} < 90$ ml/min/1.73 m², and the uNGAL and UNC values between these two groups were comparable. In view of the small number of patients with $eGFR_{(CKD\text{-}EPI40)} \geq 120$ ml/min/1.73 m², no glomerular hyperfiltration assessment group is formed, therefore we cannot assess the role of the marker in the diagnosis of this early stage of renal impairment in DM I. Additionally, we believe that the analysis used in the study had unsatisfactory analytical characteristics in the determination of low NGAL values, therefore it is not applicable for the assessment of glomerular hyperfiltration, as healthy children also have pNGAL values below the measurement range of the method (LLOQ). However, our results indicate the possible perspective of the marker, but not of the analysis, for the assessment of the glomerular hyperfiltration stage in patients with DM. In support of this finding are also data from the study of Fu WJ et al. (2012), which found significantly lower levels of pNGAL in patients with DM II with glomerular hyperfiltration compared to controls (12.0 vs 21.7 ng/ml). They explain the results obtained with the haemodynamic changes leading to a higher clearance of plasma NGAL at this early stage of renal impairment in DM. Sołtysiak J et al. (2014) found a significant difference in circulating NGAL values between patients with DM I with eGFR >135 ml/min/1.73 m², both compared to patients with preserved glomerular filtration and to the control group (111.3 vs 129.5 vs 151.3 ng/ml). The authors highlighted the importance of glomerular hyperfiltration on pNGAL concentration, but pointed to the need for further studies to confirm the results obtained.

The earliest manifestation of DKD in DM I is the glomerular hyperfiltration due to afferent arteriolar vasodilation as a result of the hyperglycaemia-induced vasoactive mediators, but an important role is also played by the tubular damage, which potentiates the effect of tubular-glomerular feedback. The Gold Standard in the determination of GFR is to measure the clearance of the exogenous substance inulin during a continuous intravenous infusion. This method is invasive, requires a lot of work, and is not routinely applied, especially in paediatric patients. For this reason, calculation formulas relying on the clearance of endogenous markers, which are eliminated from the body entirely by glomerular filtration, are most commonly applied for the determination of GFR. The updated formula of “Bedside Schwartz” from 2009 is recommended for the calculation of eGFR

in children. This includes serum creatinine concentration as measured by a method traceable to ID-MS and the height of the subject. The formula was validated for children with DKD with a GFR range of 15 to 75 ml/min/1.73 m². The eGFR values obtained from this formula have a P30 ~ 80%, therefore there is an 80% chance that the results will be within ± 30% of the real GFR. All of this points to limitations in the use of the 'Bedside Schwartz' formula in the early phases of DKD when one would expect to see glomerular hyperfiltration nd GFR values of >120 ml/min/1.73 m². Alternative formula is suggested by Björk J et al. (2021), called CKD-EPI40. CKD-EPI40 was validated among a cohort of 4005 children and was based on the CKD-EPI equation, but with age- and gender-adjusted serum creatinine, adjusted for age 40. In the data derived from Björk J et al. (2021), the CKD-EPI40 formula showed better diagnostic accuracy than the Bedside Schwartz formula, given the reference measured clearance in children with eGFR >75 ml/min/1.73m^2. Although the results are promising, it is necessary to accumulate additional data to assess the clinical relevance of the CKD-EPI40 formula in different paediatric populations before introducing it into routine practice. The testing and validation of biomarkers that are affected by changes in glomerular filtration is also a possible alternative in patients with DM I. NGAL is a low molecular mass protein that is freely filtered through the glomerular filtration barrier but is largely reabsorbed into the proximal renal tubules. Therefore, without concomitant tubular damage, the concentration of circulating NGAL should not be expected to reflect the presence of glomerular hyperfiltration. However, more and more data suggest that tubal injury is the primary disorder initiating renal dysfunction in patients with DM. A number of pathomorphological changes in the tubulointerstitium that precede or accompany pathognomonic changes in the glomerulus are described. Therefore, in the early stage of hyperfiltration of renal impairment, a disturbance in the function of the proximal tubules may also be expected in DM patients, which could result in limited reabsorption of the filtered NGAL. In this hypothetical situation, an increased GFR would result in a decrease in pNGAL concentration, but in parallel with an increase in uNGAL.

The majority of studies conducted in patients with DM I evaluated the association of pNGAL with changes in AER/ACR. In the present study, there was no statistically significant difference in pNGAL concentration between the control group and the DM I cohort, as well as between the patient groups separated by ACR. In contrast to these results, Papadopoulou-Marketou N et al. (2017) found that the mean pNGAL values in the two cohorts – children and adults with DM I – differed significantly both from each other (67.6 vs 85.2 ng/ml) and from their respective control groups (67.6 vs 24.6 ng/ml and 85.2 vs 76.1 ng/ml). The authors defined pNGAL as a biomarker for renal dysfunction showing a negative correlation with eGFR ($p<0.001$). Zachwieja J et al. (2010) found a significant difference in mean pNGAL values in children with DM I with normal AER, compared to the controls (867.4 vs 196.2 ng/ml). The authors note that normal AER in diabetics does not exclude

the presence of DKD and indicate pNGAL as a more sensitive marker than AER for assessment of renal function in children with DM (Table 35).

Table 35. Mean values/median pNGAL in patients with DM I

Study	Age CG/TG	CG	A1	A2	Units	+ ACR p<0.05	- GFR p<0.05	Method
Lacquaniti A, 2013	35/37	46.4	193.7		ng/ml	No	No	ELISA Gentofte
Zachwieja J, 2010	-/13	196.2	867.4		ng/ml	Yes	No	ELISA CycLex Co
Papadopoulou, 2017	11/14	24.6	67.6		ng/ml	No	Yes	ELISA BioPorto
Papadopoulou, 2017	27/28	76.1	85.2		ng/ml	No	Yes	ELISA BioPorto
current study	12/13	66.1[1,2]	57.7[1,3]	66.5[2,3]	ng/ml	No	Yes CKDEPI	ELISA BioPorto

[1] no significant difference between A1 and control group (p>0.05), [2] no significant difference between A2 and control group (p>0.05), [3] no significant difference between A2 and A1 (p>0.05), CG – control group, TG – target group

The present study found that only UNC values were significantly higher in the entire DM I cohort compared to the control group. Patients with DM I with elevated ACR had statistically significantly higher scores for uNGAL and UNC, both in the control group and in patient groups with normal ACR. Patients with poor glycaemic control had significantly higher UNC scores compared to both the control group and those with good control of DM I.

A statistically significant positive correlation was found between uNGAL with AER, ACR, HbA1c, BMI and triglycerides and between UNC with ACR, AER, HbA1c and triglycerides. No correlation was found between either marker with $eGFR_{(Bedside\ Schwartz)}$ and $eGFR_{(CKD\text{-}EPI40)}$.

The ROC analysis demonstrated good diagnostic effectiveness of uNGAL in differentiating patients with DM I with elevated ACR (A1 vs A2/A3) with AUC-ROC – 0.836. The diagnostic effectiveness, with which uNGAL value >47.85 ng/ml correctly identifies patients with albuminuria among children with DM I, is 86%. The diagnostic effectiveness with which the UNC value >3.86 µg/mmol correctly identifies patients with albuminuria among children with DM I, is 89%. UNC showed better diagnostic reliability than uNGAL in diagnosing patients with DM I with pathological albuminuria. The use of UNC on its own statistically significantly differentiates children with MD I with ACR >3 g/mol, and pNGAL does not significantly improve this predictive value of the marker. The increase in UNC by 1 µg/mmol in a patient with DM I increases 2.1-fold the probability that this person might have an ACR >3g/mol. Data show that uNGAL and UNC are effective markers for differentiating patients with DM I with elevated ACR and for detecting DKD.

Data from literature sources indicating the predictive value of uNGAL and UNC in the diagnosis of DKD, are also in accordance with our results. Hafez MH et al. (2015) studied the role of uNGAL in the diagnosis of DKD in children with DM I and introduced significantly higher results in patients with pathological albuminuria compared to those with normal albuminuria and to the control group

(39.14 vs 15.69 vs 5.66 ng/ml), a significant correlation between uNGAL and ACR values was found (p=0.001), but not with eGFR. Their cut-off value of uNGAL is 11.75 ng/ml with AUC-ROC-0.821 in the distinction of patients with DM I with ACR >3 g/mol. Most of the studies conducted in patients with DM I evaluated the role of the marker to diagnose renal impairment in normal AER/ACR. The derived cut-off values distinguish healthy children from those with DM I with normoalbuminuria. Zachwieja J et al. (2010) found a significant difference in mean uNGAL values in children with DM I with normal AER versus controls (420.0 vs 156.5 ng/ml). Ucakturk A et al. (2009) described a significant difference in UNC values between DM I patients with normal AER compared to the reference group (33.0 vs 13.3 ng/ml), but found no significant correlation between UNC and AER and eGFR (Table 36, Table 37).

Table 36. . Mean values/medians of uNGAL and UNC in patients with DM I

Study	Age CG/TG	Indicator	CG	A1	A2	A3	Units	+ ACR p<0.05	- GFR p<0.05	Method
Lacquaniti A 2013	35/37	uNGAL	6.5	25.5			ng/ml	Yes	No	ELISA Gentofte
Zachwieja J, 2010	-/13	uNGAL	156.5	420.0			ng/ml	Yes	No	ELISA CycLex Co
Yıldırım ZY, 2015	11/12	uNGAL	21.4	92.4[2]	145.9[2]		ng/ml	Yes	-	ELISA BioVendor
Hafez MH, 2015	12/14	uNGAL	5.7[1]	15.7[1]	39.1		ng/ml	Yes	No	Immunonp helometry
Kamel A, 2019	-	uNGAL	103.9[1]	127.2[1]	164.3		ng/ml	-	-	ELISA -
current study	12/13	uNGAL	13.7[1]	12.0[1]	41.4		ng/ml	Yes	No	ELISA Bioporto
Ucakturk A, 2009	13/13	UNC	13.3	33.0			ng/mg	No	No	ELISA BioVendor
Nielsen SE, 2010	51/56:54:49	UNC	74.0	146.0[2]	222.0[2,3]	261.0[3]	pg/mmol	Yes (A3)	-	ELISA Bioporto
Yıldırım ZY, 2015	11/12	UNC	32.1	121.3[2]	104.4[2]		ng/mg	No	-	ELISA BioVendor
current study	12/13	UNC	1.47[1]	1.64[1]	8.49		µg/mmol	Yes	No	ELISA Bioporto

[1] no significant difference between A1 and the control group (p>0.05), [2]no significant difference between A2 and A1 (p>0.05), [3] no significant difference between A3 and A2 (p>0.05). CG -control group, TG – target group

Table 37. Diagnostic effectiveness of uNGAL and UNC in distinguishing patients with ACR >3g/mol among subjects with DM I

Study	indicator	cut-off	units	DSen	DSpec	LR+	LR-	PPV	NPV	DE
Hafez MH, 2015	uNGAL	11.7	ng/ml	82%	67%	2.48	0.27			
Kamel A, 2019	uNGAL	132.5	ng/ml	100%	63%	2.70	<0.01			
current study	uNGAL	47.8	ng/ml	52%	100%	>52.0	0.48	100%	84%	86%
current study	UNC	3.86	µg/mmol	67%	98%	35.1	0.34	93%	88%	89%

DSen – diagnostic sensitivity, DSpec – diagnostic specificity, LR+ – positive probability ratio, LR- – negative probability ratio, PPV – positive predictive value, NPV – negative predictive value, DE – diagnostic effectiveness

Data from other studies are also consistent with our results for higher UNC levels in patients with poor DM I control. Hafez MH et al. (2015) reported a significant positive correlation between uNGAL and duration of diabetes, HbA1c and dyslipidaemia. Sołtysiak J et al. (2014) found a significant difference in UNC values between patients with DM I divided by glycaemic control, with values increasing progressively in the four groups from ideal to poor control.

DM I is most often diagnosed with its occurrence, allowing better monitoring and diagnosis of DKD. It is recommended that DKD screening be initiated 5 years after diagnosis via AER/ACR testing. Biomarkers that correlate with albuminuria are therefore suitable for the detection of DKD. Data from our study show that the uNGAL and UNC markers possess the necessary diagnostic effectiveness to differentiate patients with ACR >3 g/mol among individuals with DM I. This outlines the promising role of uNGAL and UNC in the diagnosis of DKD among patients with DM I (Table 46). It was found that the values of uNGAL and UNC correlate with the severity of albumin excretion (ACR) and with markers evaluating the control of DM – HbA1c and triglycerides, and therefore may be a promising marker for assessing the risk of progression of DKD in patients with DM I. Table 38 presents the cut-off values defined in the present study for DKD in patients with DM I (ACR >3 g/mol in patients with DM I) (Table 38).

Table 38. NGAL - Cut-off values for DKD – ACR >3 g/mol in patients with DM I

Indicator	cut-off	units	DSen	DSpec	LR+	LR-	PPV	NPV	DE
uNGAL	47.85	ng/ml	52%	100%	>52.0	0.48	100%	84%	86%
UNC	3.86	µg/mmol	67%	98%	35.1	0.34	93%	88%	89%

Our study found that the urinary concentration of NGAL was also dependent on IPP and the presence of leukocyturia. In 5 of the targeted patients with DM II ($\bar{x}$ - uNGAL=248.76 ng/ml and UNC=47.87 µg/mmol) and in 3 with DM I, significant leukocyturia was found and were excluded from the subsequent data processing ($\bar{x}$ - uNGAL=128.60 ng/ml and UNC=12.23 µg/mmol). The results obtained for uNGAL and UNC are significantly higher than the defined reference limits. NGAL is an acute phase protein whose concentration in the urine increases with UTIs due to its release from activated neutrophils and its increased expression by damaged tubular cells. Renal epithelial cells mainly secrete the monomer formulation, while neutrophils mainly release the dimeric formulation. Urinary tract infections (UTIs) are a common complication in patients with DM and may lead to increased uNGAL without the presence of DKD. Currently used antibodies in an immunoassay cannot distinguish the monomer form from the dimeric form of NGAL, which reduces the specificity of the marker in the diagnosis of DKD. Therefore, when interpreting the results of uNGAL and UNC, the influence of leukocyturia and UTIs on the marker values and ratio must be taken into account.

Results in this study are consistent with data in the literature that present NGAL as a marker with a good diagnostic profile in the diagnosis of DKD. However, the criteria for DKD adopted by us are the changes in laboratory parameters eGFR and ACR, therefore we cannot assess the role of NGAL as an earlier marker for the occurrence of renal dysfunction compared to these classic indicators of renal impairment, as well as the role of NGAL as a marker for DKD in patients with non-albuminuric phenotype of DKD. It is necessary to monitor a selected cohort of patients with DM with normoalbuminuria and normal eGFR in order to evaluate the predictive value of NGAL as an early marker in the development of DKD and its possible advantage over ACR and eGFR. In addition, we take into account a number of limitations and limiting factors, such as the analytical characteristics of the analysis when determining low NGAL values, the possible variability in albumin excretion, because ACR is reported once, and the small number of patients with DM I with laboratory data for renal impairment.

VI. CONCLUSIONS

Based on our analysis, we formulate the following conclusions:

1. Data from the verification of immunoturbidimetric analysis for determination of NGAL of the company Bioporto with a biochemical analyzer ADVIA 1800 show very good analytical characteristics corresponding to national and international standards. The test demonstrated better analytical reliability in determining high versus low concentrations of the analyte and failed to accurately detect concentrations of NGAL<12 ng/ml.
2. The presentation of the results for uNGAL as a ratio to uCreatinine allows for better comparability of results as well as more reliable results under non-standard conditions for collection of biological material, thus compensating for variations from daily fluctuations in urine osmolarity.
3. When establishing the reference limits for the Bulgarian adult population, a correlation between the concentration of pNGAL and age was established. Said correlation has to do with age-defined changes in eGFR.
4. When establishing the reference limits for the Bulgarian population in adults, there was a statistically significant difference between the two sexes regarding the UNC values. Women scored higher for UNC, which is parallel to lower urinary creatinine levels.
5. In patients with DM II without DKD, there was a statistically significant difference in UNC values between the two sexes. Women scored higher for UNC, which is parallel to lower urinary creatinine levels.
6. In patients with DM II, the concentration of pNGAL correlated with the decrease in eGFR, while the changes in uNGAL and UNC values correlated with the increase in ACR. The use of pNGAL on its own with good diagnostic efficiency differentiates patients with DM II with reduced glomerular filtration, while UNC differentiates patients with very good efficiency from those with increased ACR.
7. In patients with DM II, it was found that of the three indicators evaluated – pNGAL, uNGAL and UNC – the changes in UNC values show the highest prognostic value for diagnosis of DKD. pNGAL did not improve the prognostic value of UNC as a marker for diagnosis of DKD. The use of sex-differentiated cut-off UNC values on their own has statistical significance and showed very good diagnostic effectiveness in distinguishing patients with renal impairment among persons with DM II.
8. In patients with DM II, pNGAL was found to improve the prognostic value of UNC in staging DKD. When taking into account the changes in the values of pNGAL and UNC, an effective prognosis and stratification of DKD is achieved in patients with DM II.

9. In children with DM I, renal impairment was observed in a small number of patients, in accordance with the relatively recent onset of DM I.
10. In patients with DM II, the concentration of pNGAL correlated with the decrease in eGFR, while the changes in uNGAL and UNC values correlated with the increase in ACR. The use of UNC alone is very effective in distinguishing patients with DM I with increased ACR.
11. In patients with DM I, it was found that of the three indicators evaluated – pNGAL, uNGAL and UNC – the changes in UNC values show the highest prognostic value for diagnosis of DKD. pNGAL did not significantly improve the prognostic value of UNC as a marker for diagnosis of DKD. The use of the defined cut-off UNC value on its own has statistical significance and showed very good diagnostic effectiveness in distinguishing patients with renal impairment among persons with DM I.
12. In patients with DM I, it was found that the use of UNC alone has good prognostic value and best clinical relevance in prognosis and staging of DKD.
13. When interpreting the results for uNGAL and UNC, it is necessary to take into account the influence of UTIs and leukocyturia.

VII. CONTRIBUTION

Theoretical contributions

1. The high analytical reliability of the NGAL Test™ of the company Bioporto in determining the concentration of NGAL in plasma and urine was confirmed.
2. The presentation of results for uNGAL as a ratio to uCreatinine was found to allow for better comparability of results as well as for obtaining more reliable results when using spot urine.
3. It was found that there is a male/female difference in individuals with renal impairment regarding UNC values.
4. It was found that in patients with DM, the concentration of pNGAL correlated with the decrease in eGFR, while the concentration of uNGAL and UNC correlates to the increase in ACR.
5. It was found that in patients with DM II, the use of UNC alone is an effective marker for diagnosis, while the simultaneous use of pNGAL and UNC is effective for prognosis of DKD.
6. It was found that in patients with DM I, the use of UNC alone is an effective marker for diagnosis and prognosis of DKD.
7. It was also found that the concentration of uNGAL is dependent on UTIs and leukocyturia.

Practical and applicable contributions

1. This is the first time that an analytical verification of immunoturbidimetric analysis for determination of NGAL is performed in Bulgaria
2. For the first time in Bulgaria, sex-differentiated referential limits of pNGAL, uNGAL and UNC have been established in adults, determined by the clinically applicable immunoturbidimetric analysis.
3. For the first time in Bulgaria, referential limits of pNGAL, uNGAL and UNC were determined in children, as determined by the clinically applicable immunoturbidimetric analysis.
4. For the first time in a Bulgarian cohort of patients with DM II, the prognostic role of NGAL as a marker for diagnosis of DKD has been evaluated. Cut-off values were defined, which can be applied in routine practice.
5. For the first time in a Bulgarian cohort of patients with DM II the prognostic role of NGAL as a prognosis marker of DKD was evaluated.
6. For the first time in a Bulgarian cohort of patients with DM I, the prognostic role of NGAL as a marker for diagnosis of DKD has been evaluated. Cut-off values were defined, which can be applied in routine practice.
7. For the first time in a Bulgarian cohort of patients with DM I the prognostic role of NGAL as a prognosis marker of DKD was evaluated.

VIII. SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION

1. Shefket S, Bocheva Ya. PREDICTIVE ROLE OF NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN AS AN EARLY MARKER OF RENAL IMPAIRMENT IN PATIENTS WITH DIABETES MELLITUS. *Current nephrology*. 2021. XV.1:51-61.
2. Shefket S, Bocheva Y. REFERENCE LIMITS FOR THE BULGARIAN POPULATION OF NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN MEASURED BY IMMUNOTURBIDIMETRIC ANALYSIS. *Nephrology, dialysis and transplantation*. 2021; 4:30-39.

PARTICIPATION IN SCIENTIFIC FORUMS RELATED TO THE DISSERTATION

1. Shefket S, Bocheva Y, Popcheva G, Boyadzieva M. uNGAL - an Early Marker for Kidney Injury in Patients with Type I Diabetes Mellitus. *Clinica Chemica Acta*. 2019.V.493:S294-295.
2. Shefket S, Bocheva Y, Popcheva G, Galcheva S. Association between NGAL and glycemic control in patients with type I diabetes mellitus. *Turkish Journal of biochemistry* 2019. V.44(S3).

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