Original Research Paper

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EARLY DIAGNOSTICS OF DIABETIC NEPHROPATHY

Endocrinology

KEYWORDS: Diabet; CKD; albuminuria; glomerular filtration rate; cystatin C ; albumin: creatinine

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ABSTRACT

This study was conducted in 153 healthy volunteers (controls) and 314 patients with diabetes mellitus type 2 (Dm2), assigned into two subgroups (with and without hypertension) to establish the diagnostic sensitivity and specificity of the AU (A1, A2, A3) and GFR in different stages of DN.

In diabetics and controls we performed a detailed clinical examination, analysis of the parameters: glucose, glycosylated hemoglobin, cystatin C, in blood, albumin, albumin percentage of the total protein, ACR, PCR, creatinine in the urine; GFR with creatinine and cystatin C.

In the control group consisting of 153 volunteers the albumin concentration in the urine is of the A1 albuminuria type, below 30 mg / l. The albumin percentage in the urine of the total protein is X = 26, ACR X = 2.47, PCR X = 10.5. Changes in most of the parameters are more pronounced in the subgroup 2 compared subgroup 1. 77 patients of the first subgroup - 50.7% are with albuminuria A1, with albuminuria A2 - 71 patients - 46.7% and with albuminuria A3 - only 4 patients - 2.6%, and in the second subgroup there are 71 patients (43.8%), 85 patients (52.5%) also with albuminuria A3 - 6 patients (3.7%) respectively..

Early diagnosis of DN requires that laboratory diagnostics begins as soon as diabetes type 2 is registered including AU, PU, ACR, PCR, albumin percentage of total protein and GFR. To estimate GFR it is best to use the combined formula with creatinine and the new biomarker cystatin C. The hypertension in diabetics increases the likelihood of more frequent and severe kidney damage, demonstrated by stronger increase in protein parameters and stronger reduction of GFR.

INTRODUCTION

Diabetes is a socially significant disease, which tends to increase continuously throughout the whole world, as well as in our country. More than 33 million diabetics, aged between 20 and 79 have been registered in Europe. In Bulgaria, they amount to 520 000 and this number is constatntly increasing. The diabetes affects individuals from earlier age [1, 2, 3]. One of the most frequent and severe complications of diabetes is the diabetic nephropathy (DN), which affects almost every other patient in 10-15-20 years' time after the disease was first dignosed. Unfortunately, DN diagnosis is often delayed due to the lack of clinical manifestation. After its occurrence and incorrect treatment it progresses to chronic renal failure (CRF) which leads to haemodialysis and/or transplantation. Diabetes is the reason for 30-40% of patients to develop end-stage renal disease (ESRD) [4, 5]. The laboratory diagnostics of DN dates back to the late 18th century, when proteinuria (PU) in diabetes was first reported. In 1930 Kimmelstiel and Wilson described the classic lesions of nodular glomerulosclerosis in diabetes associated with proteinuria (PU) and hypertension [6]. For a long time the diagnosis of DN has been based only on proteinuria. In some cases, however, this test alone is not sufficient for the early detection of DN. In the early 1980s studies from Europe showed that small amounts of albumin in the urine, undetectable by conventional methods, were predictive of later development of proteinuria A2 in patients with diabetes mellitus type 1 and type 2. This stage of kidney damage is called microalbuminuria or incipient nephropathy. It turned out that albuminuria is an earlier and more specific indicator than proteinuria. Microalbuminuria and albumin: creatinine ratio in urine (ACR) successfully complete and expand each other in the early diagnosis of DN [7, 8, 9, 10]. Dissociation between albuminuria respectively proteinuria and reduced glomerular filtration has been registered in some of the diabetics. For many years the GFR has been the basis for classification of CKD in 5 stages (I, II, III, IV and V). Since the beginning of 2012, according to the recommendations of KDIGO and NKF, CKD has been subdivided into 6 stages (Stage III has been divided into IIIa and IIIb) and based on the three factors: **reason, GFR and albuminuria** [11, 12]. Moreover, albuminuria has been classified and named in a new manner, i.e. albuminuria A1, with normal or very slightly increased albumin concentration of up to 30 mg/l; albuminuria A2, also known as microalbuminuria, with albumin concentration in the range of 30 to 299 mg/l; albuminuria A3 or macroalbuminuria with albumin concentrations above > 300 mg/l. The occurance of albuminuria A2 and GFR <60 mL/min/ 1.73 m^2 lasting for more than three months signifies to chronic renal failure (CRF). Although the exogenous marker inulin can be considered the "gold standard" for assessing the GFR, its use and the one of other exogenous markers is limited due to various reasons like invasiveness, cost, adverse allergic reactions and time to reach a steady state. Recently cystatin C, a new endogenous marker, has been introduced [13, 14, 15]. The advantages and disadvantages of cystatin C in comparison to creatinine are still a subject of discussion. There has been a dispute on the modified and new formulas used to estimate the GFR - only creatinine or cystatin C or with both biomarkers (16, 17). The purpose of this study is to determine the diagnostic sensitivity and specificity of albuminuria (A2 and A3), proteinuria and GFR in 153 controls and 314 diabetic patients (with or without hypertension).

MATERIALS AND METHODS

Controls and diabetics

This study was conducted from the beginning of 2008 until the end of 2014 in the clinical laboratory Ramus, Sofia. During this period a total of 467 adult volunteers were monitored. The clinically healthy individuals (the control group) were 153; 314 patients were with diabetes mellitus type 2 (152 without hypertension and 162 with hypertension). We obtained informed consent from each participant to be included in the study. The criteria for selecting participants from the reference group are in line with the recommendations of the IFCC. As for the patients, they were diagnosed with diabetes mellitus type 2 (DM2). For checking the clinical parameters of all participants a full clinical examination, standard urineanalysis, haematological and biochemical tests were carried out. In addition, assessments of markers for hepatitis B, C and HIV, blood pressure and electrocardiogram were performed.

METHODS

During the laboratory tests the principles of Good Clinical Laboratory Practice (GCLP) were observed. The following parameters were tested: glucose, glycosylated hemoglobin, cystatin C, creatinine, uric acid, total cholesterol, HDL-C, LDL-C and triglycerides in the blood serum; tests were also performed to establish the values of albumin, albumin percentage of the total

protein, total protein, albumin:creatinine ratio (ACR), total protein: creatinine ratio (PCR), creatinine in the urine, ECG, blood pressure, etc. General urine is tested automatically with test strips. The amount of albumin in the urine is determined with the help of the immunoturbidimetric method, using the Mikroalbumin MULTI-GENT test of Architect c8000 system (Abbott Laboratories Inc.) while the turbidimetric benzethonium chloride method of Abbott Laboratories Inc. was used to establish the amount of total protein. The creatinine in both the serum and the urine is determined with the kinetic Jaffe method. The cystatin C in the serum is analyzed with the PETIA method (particle-enhanced turbidimetric immunoassay). The other laboratory parameters are determined by using the generally accepted methods. To estimate the GFR we used the following optimized equations [11,12, 21]:

1. MDRD (number 1) with creatinine only eGFR (mL/min/1.73 m2) = 175 × (SCR)-1, 154 × (age)-0, 203 0.742 (if female)

2. CKD-EPI(number 2) with creatinine only

eGFR (mL/min/1.73 m2) =144 (sCr/0.7)-0.329 x (0.993)age (for females with creatinine ≤ 0.7)

eGFR (mL/min/1.73 m2)=144x (sCr/0.7) -1.209 x (0.993)age (for females with creatinine >0.7)

eGFR (mL/min/1.73 m2) =141 x (sCr/0.9) -0.411 x (0.996)age (for males with creatinine ≤ 0.9)

eGFR (mL/min/1.73 m2) =141x (sCr/0.9) -1.209 x (0.996) age (for males with creatinine > 0.9)

3. CKD-EPI(number 3) with cystatin C only (for males and females) with cystatin C ≤ 0.8)

eGFR (mL/min/1.73 m2) =133 x (sCys/0.8) -0.499 x (0.993)age x 0.932 (for females with cystatin C ≤ 0.8)

eGFR (mL/min/1.73 m2) =133 x (sCys/0.8) -1.328 x (0.993)age x 0.932 (for males with cystatin C > 0.8)

4. CKD-EPI(number 4) with creatinine and cystatine C
eGFR (mL/min/1.73 m2) =130 x (sCys/0.7) -0.248 x (sCys/0.8)-0.375 x 0.995age (for females with creatinine ≤ 0.7 and cystatine C≤ 0.8)

eGFR (mL/min/1.73 m2) =130 x (sCr/0.7) -0.248 x (sCys/0.8)-0.711 x 0.995age (for females with creatinine > 0.7 and cystatine C> 0.8)

eGFR (mL/min/1.73 m2) =135 x (sCr/0.9) -0.207 x (sCys/0.8)-0.375 x 0.995age (for males with creatinine ≤ 0.9 and cystatine C≤ 0.8)

eGFR (mL/min/1.73 m2) =135 x (sCr/0.9)-0.207 x (sCys/0.8)-0.711 x 0.995age (for males with creatinine > 0.9 and cystatine C> 0.8)

RESULTS

The data from the anthropometric characteristics and the variation analysis is presented in Table 1. 153 volunteers from 20 to 77 years of age have been included in the control group. The mean values estimated by us and SD (Table 2) for the parameters examined in controls are close to normal. Albumin concentration in the urine in all controls is of albuminuria A1 type, below 30 mg/l. The percentage of albumin in the urine of the total protein was 26.1. We have found significant gender differences for albumin in urine and ACR with higher values in females than males (13.48 mg/l vs. 12.11 mg/l, and ACR females 2.69 vs. 2.24 males, $p < 0.05$). We estimated the GFR using four formulas in controls and diabetics to determine which equation is best. The GFR is similar when estimated with various updated formulas and in the controls it has an average value between 76 and 109 mL / min / 1.73 m^2. It is slightly higher in males when using the cystatin C formula. Cystatin C shows a higher correlation with the GFR when estimated with the fourth equation ($r = - 0.688$, $p < 0.05$) and ACR than the serum creatinine ($r = - 0.360$, $p < 0.05$). The control group size and the values obtained for their individual parameters enable us to assume that these values can be accepted as reference for the country. They can also be used for comparing the patients' group parameters. We allocated the studied 314 patients with type 2 diabetes in two subgroups (the first subgroup (1) without hypertension and the second subgroup (2) with diabetes and with hypertension). Finding patients with diabetes that do not manifest high blood pressure (> 140 / 80mmHg) was a challenge, regardless of the duration of the disease and multiple measurements of the blood pressure. The data from the anthropometric characteristics and the variation analysis is presented in Table 1 and 2. The mean values of the anthropometric characteristics are somewhat higher in the diabetics with and without hypertension in comparison with the control group, including weight, age and BMI (Table 1). The diabetics in subgroup 1 had different disease duration: a 2-year-long disease history (8 patients, 5.3%), up to a 5-year-long disease history (77 patients, 50.7%), up to a 10-year-long disease history (64 patients, 42.1%) and up to a 12-year-long disease history (3 patients, 1.9%). The second subgroup consisted of 162 patients with diabetes type 2. They developed hypertension (> 140/80 mmHg) at a different point after having been diagnosed with diabetes. Their diabetes had 1-year-long history (2 patients, 1.2%), up to a 5-year-long history (85 patients, 52.5%), up to a 10-year-long history (66 patients, 40.7%), up to a 15-year-long history (8 patients, 4.9%) and up to a 16-year-long history (1 patient, 0.61).

Table 1: Studied contingent - controls and diabetics

Group	Number (total)	Males	Females	Age	Weight (average)	Height (average)	BMI (average)
Controls	153	63	90	20 - 77	65.74	165.71	23.91
Diabetics without hypertension	152	80	72	24 - 82	74.18	167.34	26.33
Diabetics with hypertension	162	83	79	18 - 86	67.31	168.60	27.71

Table 2: Variation analysis of the studied parameters in the control group. diabetics without hypertension and diabetics with hypertension

Group	Control group					Diabetics without hypertension					Diabetics with hypertension				
PARAMETER		SD	Median	Min	Max		SD	Median	Min	Max		SD	Median	Min	Max
Cystatin C (mg/L)	0.74	0.11	0.73	0.50	1.02	1.03	0.28	0.98	0.51	1.98	1.00	0.34	0.95	0.53	2.21
Urine creatinine (mmol/L)	7.85	5.24	6.07	0.65	21.41	4.53	2.88	3.65	1.09	14.93	4.61	3.32	2.75	1.15	16.83
Albumin in urine (mg/L)	12.92	4.05	13.00	5.00	22.00	51.43	67.46	28.50	10.00	364.00	55.88	37.97	52.00	9.00	136.00
Total protein in urine(mg/L)	54.39	19.24	54.50	16.00	109.0	118.01	145.68	72.00	19.00	825.00	125.11	43.06	107.00	26.00	209.00

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Percentage of albumin from the total protein in urine (%)	26.14	11.85	23.08	6.85	68.18	43.70	16.68	41.75	16.50	97.20	49.08	17.52	35.31	11.63	82.50
ACR (mg/mmol)	2.47	1.91	2.01	0.33	9.32	15.25	22.31	7.60	1.00	137.66	17.31	30.04	19.49	4.50	84.35
PCR (mg/mmol)	10.50	8.74	8.20	1.87	53.27	35.87	51.44	21.81	2.18	373.21	37.05	37.64	35.80	8.14	118.87
Cholesterol (mmol/L)	4.72	0.60	4.89	3.21	5.99	5.98	1.11	5.91	3.90	9.97	6.49	1.97	6.12	3.40	15.20
Triglycerides (mmol/L)	1.32	0.37	1.31	0.55	2.00	2.04	0.85	1.97	0.59	4.81	1.14	0.24	1.14	0.54	2.31
HDL – C (mmol/L)	1.15	0.16	1.13	0.85	1.79	1.07	0.24	1.00	0.62	1.98	4.22	1.85	3.87	1.31	11.92
LDL – C (mmol/L)	2.62	0.44	2.71	1.14	3.65	3.72	1.00	3.61	1.90	7.11	1.91	0.88	1.65	0.47	4.65
Glucose (mmol/L)	4.93	0.73	5.12	3.06	6.33	7.28	2.28	6.96	3.70	14.20	6.61	2.43	5.99	3.21	15.20
Glycosylated hemoglobin(%)	5.37	0.44	5.33	4.21	6.32	7.17	1.41	6.98	4.97	12.30	7.08	1.72	6.50	4.50	13.20
Creatinine (µmol/L)	83.47	15.44	80.00	56.00	120.00	86.97	21.07	86.00	56.00	169.00	97.64	14.53	74.15	49.00	129.90
Uric acid (µmol/L)	256.92	84.57	251.00	56.00	430.00	292.74	74.82	292.50	103.00	465.00	316.79	84.78	307.00	139.00	504.00
eGFR (MDRD) with creatinine only (mL/min/1.73m^2)	76.24	14.15	72.36	59.12	132.33	73.55	17.31	74.17	28.88	120.91	81.23	18.57	80.50	41.26	129.70
eGFR (CKD-EPI) with creatinine only (mL/min/1.73m^2)	84.82	15.29	81.27	54.94	129.42	79.51	19.46	80.42	29.47	120.72	85.11	18.09	84.79	43.48	131.80
eGFR (CKD-EPI) with cystatin C only (mL/min/1.73m^2)	109.81	15.05	111.31	70.55	139.88	78.97	25.06	79.66	31.81	137.88	82.67	25.30	82.41	27.58	146.68
eGFR (CKD-EPI) with creatinine and cystatin C (mL/min/1.73m^2)	97.52	13.20	96.09	63.56	137.95	78.52	20.07	78.34	31.00	135.66	83.53	20.27	83.36	40.51	144.20

In all patients from this group, the hypertension was registered from several months to 5 years after the diabetes diagnosis. We chose to follow diabetics with a relatively short history of diabetes. The average values of all studied biochemical parameters in both subgroups of diabetics were higher in comparison to the controls (see table 2), while the GFR – were lower. Changes in most of the parameters were more pronounced in subgroup 2 rather than in subgroup 1. In the first subgroup 77 patients (50.7%) were with albuminuria A1 (norm), 71 patients (46.7%) with albuminuria A2 and only 4 patients (2.6%) with albuminuria A3. As for the second subgroup (diabetics with hypertension) there were 71 patients (43.8%) with albuminuria A1, 85 patients (52.5%) with albuminuria A2 and 6 patients with albuminuria A3 (3.7%). In the first subgroup there were 106 patients (69.7%) with normal proteinuria of up to 150 mg/l, 40 patients (26.3%) with proteinuria up to 500 mg/l and only 6 patients had TP > 500 mg/l (3.9%). In the second subgroup there were 75 patients (46.3%) with total protein up to 150 mg/l, 76 patients (46.9%) up to 0.5 g/l and 11 patients (6.8%) with > 0.5g/l. From the diabetics without hypertension 75 (49.3%) were with albuminuria (A2 + A3) and 87 (53.7%) diabetics with hypertension. In contrast to the controls the excretion of albumin and total protein in the urine of all diabetics was higher in males (56.06 mg/l and 127.23 mg/l) compared to females (46.42 mg/l and 107.78 mg/l)). We have registered pathological albuminuria (A2 and A3) without proteinuria in 33 (10.5%) of them.

In both subgroups albuminuria A2 and A3 were observed more frequently in elderly patients (r = 0.270) and in those with older diabetes history (r = 0.535). We registered a statistically significant increase of parameters ACR, PCR and albumin percentage of total protein ($p < 0.05$). Compared to the control group%, the increase in the first and second diabetics subgroup was: for ACR 6.17 and 7.0 times; for PCR over 3.4 and 3.5 times; and for the albumin percentage of total protein 1.67 and 1.87 times. ACR and PCR are statistically higher in the females in the two groups. Albuminuria, proteinuria and albumin percentage of the total protein, ACR and PCR are more frequent and with more pronounced changes in the diabetic patients with hypertension ($p < 0.05$). The serum concentration of creatinine and cystatin C was significantly increased in both subgroups in comparison to the controls and the increase was greater in diabetic patients with albuminuria A2 plus A3 vs. those with A1: for creatinine 88.12 + 16.3 µmol/l against 105.15 + 23.4 µmol/l and for cystatin C 0.96 + 0.26 mg/l compared to 1.12 + 0.24 mg/l). Calculated as a percentage the increase is more common for cystatin C (36%) compared with creatinine (27%). Compared to the controls the parameters of the lipid profile in the two subgroups were increased significantly in diabetics with hypertension: total cholesterol at cut-off> 6 mmol/l in 40.8%; LDL-C at cut-off> 3.65 mmol/l in 46.7% and HDL-C at cut-off <1.0 mmol/l in 40.6%. We observed higher values in 59.7% and 60% respectively of all diabetics given that the accepted cut-off for HbA1c was <6.5% and glucose> 6.3 mmol/l. In the general group of diabetics, the excretion of albumin showed quite strong positive correlation with the TP ($r = 0.814$, $p < 0.001$), with the disease history ($r = 0.733$, $p < 0.001$), with the ACR ($r = 0.763$, $p < 0.001$) with the cystatin C ($r = 0.514$, $p < 0.01$) with the serum creatinine ($r = 0.232$, $p < 0.05$) and the glycated hemoglobin ($r = 0.424$, $p < 0.01$). The main value of the GFR estimated with the four formulas was lower compared to the controls in both subgroups, with the greatest percentage of decrease in the equation with cystatin C (38%) followed by the combined formula with creatinine and cystatin C (24%). The correlation coefficients of the GFR estimated with the four formulas were inversely proportional to any protein parameters. Values of the negative coefficients were the highest between the GFR and the duration of disease ($r = -0.716$, $p < 0.05$) as well as albumin in the urine ($r = -0.471$, $p < 0.05$) and HbA1c ($r = -0.449$, $p < 0.05$). The results of the GFR according to the classification of CKD are presented in Table 3. We don't have patients who fall in Group IV and V of CKD. The GFR in the controls is quite unidirectional with formula 2, 3 and 4 except for the MDRD equation. Only with this equation 4 (2.6%) the controls demonstrated GFR <60 mL / min / 1.73 m^2 while in the other formulations (2, 3 and 4) all controls showed GFR> 60 mL / min / 1.73 m^2. Both subgroups of diabetics showed changes in the GFR with different formulas with similar trends. We accepted the results of the combined formula with creatinine and cystatin to be the most reliable of all the formulas estimating the GFR. It was with this formula that 32 (21.1%) diabetic patients without hypertension and 21 (12.9%) hypertensive diabetics were with GFR <60 mL / min / 1.73 m^2. We registered more often albuminuria A2 and A3 and an increase in the ACR in comparison with the GFR <60 mL / min / 1.73 m^2. Most of them were the diabetics with albuminuria A2 and A3 (166 patients, 52.86%) of the GFR group <60 mL / min / 1.73 m^2 (53 patients, 16.9%) estimated with formula 4. All patients with albuminuria A3 were with glomerular filtration rate

<60 mL / min / 1.73 m^2 irrespective of the formula used. 30 diabetics had albuminuria A2, although the GFR was> 60 mL / min / 1.73 m^2. We registered GFR <60 mL / min / 1.73 m^2 free of albuminuria A2 or A3 and without proteinuria over 150 mg/l in 4 patients. ROC curves have been used for the diagnostic efficacy of the tested parameters. For determining the threshold values of the quantitative variables we had used the analysis of ROC curves (Table 2) with the criteria sensitivity and precision in percentage. The selected threshold value aims at high sensitivity, specificity and precision. In diabetics the ACR was a particularly useful parameter for both subgroups with an under the curve area of 0.888 and 1.0 at p <0.001, followed by the albumin in the urine with an under the curve area of 0.895 and 0.915 p < 0,001. The ROC curves for the GFR again gave priority to the combined formula with cystatin C and creatinine (under the curve area of 0.710, and 0.787 at p <0.001) compared to those with creatinine only (under the curve area of 0.509, and 0.560 at p = 0.001.

Table 3: Allocation according to levels of glomerular filtration and study groups

Formula	Group	Levels of GFR									
		15-29		30-44		45-59		60-89		90+	
		n	%	n	%	n	%	n	%	n	%
eGFR (MDRD) with creatinine only	Controls	0	0.00	0	0.00	0	0.0	117	77.50	34	22.2
	Diabetics	1	0.70	9	5.90	20	13.20	100	65.80	22	14.50
	Diabetes + AH	0	0.00	1	0.60	16	9.90	97	59.90	48	29.60
eGFR (CKD-EPI) with creatinine only	Controls	0	0.00	0	0.00	2	1.30	96	63.60	53	35.10
	Diabetics	1	0.70	7	4.60	17	11.20	78	51.30	49	32.20
	Diabetes + AH	0	0.00	1	0.60	11	6.80	80	49.40	70	43.20
eGFR (CKD-EPI) with cystatin C only	Controls	0	0.00	0	0.00	0	0.00	19	12.40	134	87.60
	Diabetics	0	0.00	11	7.20	34	22.40	61	40.10	46	30.30
	Diabetes + AH	4	2.50	14	8.60	10	6.20	70	43.20	64	39.50
eGFR (CKD-EPI) with creatinine and cystatin C	Controls	0	0.00	0	0.00	0	0.00	40	26.50	111	73.50
	Diabetics	0	0.00	4	2.60	28	18.40	72	47.40	48	31.60
	Diabetes + AH	0	0.00	3	1.90	18	11.10	78	48.10	63	38.90

Table 3 gives information about sensitivity, specificity, predictive values and precision. The parameters which best differentiated the diabetics (first / second subgroup) from the controls were ACR at cut-off 3.23 with sensitivity 94/100%, specificity 80/93%, precision 87/97%, followed by PCR at cut-off 11.35 with sensitivity 87/97%, specificity 73/82% and precision 83/90 (Table 4). In estimating the GFR with the four formulas, the sensitivity and specificity were very close, but lower than those of the protein parameters.

Table 4: Threshold values of the studied parameters and values of the criteria for validation in differentiation of diabetics (first / second subgroups of healthy individuals)

Parameter	Threshold value	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Albumin in urine (mg/L)	≥ 15.5	80/86	69/70	72/75	78/83	75/78
Total protein in urine (mg/L)	≥ 63.5	84/90	50/68	63/75	76/87	67/79
ACR (mg/mmol)	≥ 3.23	94/100	80/93	70/94	91/100	87/97
PCR (mg/mmol)	≥ 11.35	87/97	73/82	69/85	80/96	83/90
Total cholesterol (mmol/L)	≥ 5.12	80/81	63/69	69/74	77/78	72/76
eGFR (MDRD) with creatinine only (mL/min/1.73m^2)	≥ 77.53	70/75	67/68	61/62	64/66	63/65
eGFR (CKD-EPI) with creatinine only (mL/min/1.73m^2)	≥ 85.53	75/77	68/69	60/63	62/64	66/69
eGFR (CKD-EPI) with cystatin C only (mL/min/1.73m^2)	≤ 110.68	79/83	75/77	69/71	70/71	64/70
eGFR (CKD-EPI) with creatinine and cystatin C (mL/min/1.73m^2)	≤ 104.16	81/83	77/81	76/81	69/70	62/65
Cystatin C (mg/L)	≥ 0.85	80/81	59/61	64/66	71/74	69/71
Creatinine in serum (μmol/l)	≤ 90.60	77/80	53/57	50/56	52/61	55/58

DISCUSSION

According to the IDF data published in 2011 in Europe (EU-27) diabetics will increase from 33 million in 2010 to 38 million in 2030. According to WHO, the prevalence of diabetes for all age categories all over the world is expected to rise from 2.8% in 2000 to 4.4% in 2030. One of the serious complications of DM2 is DN which was discovered as early as 1950. The spread reached up to 30-40% for a period of 10-15-20 years since its discovery. About 3% of the newly diagnosed patients with diabetes type 2 demonstrate DN. After a period of 10-20 years of DM2, an average of 3% of the patients develop DN annually (3 ,4, 5, 6, 10, 18, 19). The laboratory diagnostics of DN was based on proteinuria for many years. In 1980, studies in Europe found that lower content of albumin in the urine, which was not detectable by conventional methods, predicted the later development of proteinuria in patients with diabetes. Then this stage was called microalbuminuria or incipient nephropathy. The latter was categorized in microalbuminuria stage (A2) with albumin excretion> 20 μg / min to ≤199 μg / min and macroalbuminuria (A3) with albumin excretion ≥200 μg / min.

Since the beginning of 2012, according to the recommendations of KDIGO and NKF [11, 12], the diagnosis of CKD has been based on: reason, glomerular filtration rate, albuminuria. Albuminuria was added to the GFR that was the only one used until then. Stage III has been subdivided into IIIa and IIIb. Moreover, albuminuria has been classified and named in a new way, namely albuminuria A1, with normal or very slightly increased albumin concentration of up to 30 mg/l; albuminuria A2, also known as microalbuminuria with albumin concentration ranging from 30 to 299 mg/l; albuminuria A3 or macroalbuminuria with albumin concentrations > 300 mg/l. With albuminuria A2 and GFR < 60 mL/min/1.73 m^2 for more than three months we can speak about the presence of chronic kidney failure (7, 8, 9, 11, 12, 18). We conducted this study in 153 controls and 314 diabetics because of the commonly assumed fact that the persistent

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AU was a fundamental principle marker of kidney impairment, and the GFR was an excellent measure of the capacity of kidney filtration. Albuminuria A2 and the level of GFR are a strong predictor of the beginning of CKD and risk of complications in DN. These parameters are particularly important for the detection, assessment and control of chronic kidney disease. AU A2 is rarely not-predictive of DN. Although the exogenous marker inulin is considered the "gold standard" for estimating the GFR, its use and the one of other exogenous markers is limited due to various reasons like invasiveness, cost, adverse allergic reactions, time to reach steady state, need of special equipment, etc. Despite being criticized for analytical and physiological problems like interference of methods and its dependence on muscle mass, protein intake, tubular excretion, etc., for a long period of time the exogenous markers were displaced by creatinine (19, 20, 21, 22). Recently cystatin C, a new endogenous protein marker, has been introduced (13, 14, 15, 23, 24, 25) to estimate the GFR. This biomarker is freely filtered through the glomerul without being reabsorbed and secreted by tubules and is eliminated by the kidneys only. Modified or new formulas for GFR with creatinine, cystatin C only or with both biomarkers (26, 27) are subject to studies. DN is the 5-6th leading cause for death in many developed countries (2, 3, 6, 18). The early diagnostics of DN is very important to reduce cardiovascular disease (CVD) and mortality. Avoiding the final stage of chronic kidney failure with subsequent need of hemodialysis and kidney transplantation is directly related to the early detection of kidney damage in diabetics. There is a discrepancy about the time when the assessment of laboratory markers in DM2 should start – as soon as the disease is diagnosed or 3-5 or a few years after the beginning of the disease. Furthermore, dissociation between albuminuria and GFR has been recently reported. Using the data we collected, we support the authors, who recommend that the monitoring should begin immediately after DM2 diagnosis. This is due to the fact that kidney damage, rarely though, could precede changes in albumin excretion and glomerular filtration rate. About 7% of patients demonstrate AU A2 at the very beginning of the disease. We support the opinion that monitoring for DN in diabetic patients should initially include the protein parameters in the urine, which in most cases precede the decrease in GFR. The lower sensitivity and specificity of PU requires immediate start of AU and ACR without neglecting PU and PCR.

These tests are easy, accessible and economically viable. They are accepted by the patients without a reservation because the single morning urine is taken. The advantage is that it is not necessary to collect 24-hour urine and the influence of hydration, circadian rhythms and orthostatic PU is avoided. In case of negative results, the next examination is required in a span of 6 months to 1 year. In case of a positive results, the assay should be repeated within a few weeks to 2-3 months.

In Table 2 we present the values for individual parameters in the controls group. As it is seen they are similar to the ones reported in the literature. The excretion of albumin in the urine and the ACR are slightly higher in females compared to males (13.48 + 4.31 compared to (12.11 + 3.53 and ACR females 2.69 + 1.14 vs. 2.24 + 1.18 males, $p < 0.05$). In all of the controls, AU is type A1 (normal). The results we received are in agreement with some authors stating (10,28) that albumin in clinically healthy controls is only part of the total protein reaching up to 40%, and the average value of the excreted albumin is 26.14 + 11.85%. The remaining up to 60% proteins are non-albumin, low molecular-weight plasma proteins, light chains of immunoglobulins and tissue Tamm-Horsfall proteins. The increase in the excretion of albumin is an indicator of impairment in glomerular filtration membrane. We assume that the parameters ACR and PCR are more sensitive and specific than the excretion of albumin and protein (see Table 3). In both subgroups we observed ACR at cut-off of 3.23 with 94/100% sensitivity, 80/93% specificity and 87/97% accuracy. ACR demonstrates greater sensitivity than PCR for low levels of proteinuria, therefore it should be preferred. Generally, in diabetics with and without hypertension we observed AU A1 in 47.1%, AU A2 in 49.7% and AU A3 in 3.2%. We registered AU A2 and increased ACR in diabetic patients with disease history of less than 1 year. There is a trend of higher parameters AU, ACR and PCR in case of a longer duration of the disease and higher HbA1c. These parameters are significantly higher in diabetic patients with hypertension ($p < 0.05$). Diabetic nephropathy, also known as Kimmelstiel-Wilson syndrome, nodular diabetic glomerulosclerosis and inter capillary glomertaulonephritis is a progressive kidney disease caused by angiopathy of glomerular capillaries [6, 11, 29]. It is characterized by nephrotic syndrome and diffuse glomerulosclerosis. Three main histological changes occur in the glomeruli of people with diabetic nephropathy [6.29]: firstly, mesangial expansion directly induced by hyperglycemia, perhaps by increased production of matrix or glycosylation of matrix proteins; secondly, thickening of the glomerular basal membrane (GBM); thirdly, glomerular sclerosis due to hypertension (induced by dilation of the afferent renal artery or by ischemic injury induced by hyaline narrowing of the vessels of the glomerulus). Recently, the hypothesis that GRF is an autoimmune disease involving T lymphocytes and genetic factors has been defended (6). The hypothesis that there is glomerular sclerosis in DN due to hypertension, led us to include diabetics with and without hypertension in the study. Hypertension, simultaneously with an increase in glomerular capillary pressure and metabolic disorders (e.g., dyslipidemia, hyperglycemia) may interact to accelerate kidney impairment. The data we received undoubtedly shows that in diabetic patients with hypertension the excretion of albumin and total protein, ACR and PCR, the albumin percentage of the total protein are more often and more pronounced in comparison to diabetics without hypertension with a similar age and duration of the disease (Table 3 and 4). In the available literature we could not find division of diabetics with and without hypertension. That is why we assume that this data is one of the first in terms of this issue. We traced the GFR in the controls and the diabetics because it is a classic measure of kidney function [30, 31, 32, 33, 34]. To estimate the GFR we had in mind two important prerequisites: First, to use endogenous instead of exogenous biomarkers because they are more economical, more affordable and faster, without allergic reactions. Also we included not only the well known creatinine but also the relatively new biomarker cystatin C. According to some authors cystatin C has received much attention as an alternative filtration marker with a stronger and more linear respect to risk than creatinine. These authors argue that adding cystatin C to creatinine in the estimation of GFR significantly improves classification of risk for cardiovascular disease, kidney failure and mortality [12, 23, 34, 35]. The second premise was to use updated and new formulas for estimating the GFR, with creatinine only, with cystatin C only and a combination of both markers [25, 35, 36, 37]. There is a dispute in the literature in regard to the formula used for estimating the GFR, as a basis for diagnostic strategy - in screening, for specific clinical indication, as a secondary test based on results of creatinine test or to compare the GFR estimated with creatinine. The results enable us to assume that the combined formula with creatinine plus cystatin C has a slight advantage. It is used to estimate the GFR within the entire range with the highest precision [36, 37]. It is believed that this is the equation for estimating the GFR in the future. In compliance with the results such as absolute value, SD, ROC curves, sensitivity and specificity we support the view that the combined formula is more acceptable. It eliminates the drawbacks of both markers, particularly determinants related to glomerular filtration. One of its disadvantages is the inclusion of a second marker and the rising cost of the study. This can be neglected since it leads to earlier and more accurate diagnosis of DN and the serious consequences of it. According to us the comparison of protein parameters (excretion of albumin, ACR, PCR, albumin percentage of TP) with GFR gives priority to the first ones, because they change earlier and more often and show better diagnostic effectiveness (sensitivity, specificity, accuracy supported by data from ROC curves). In patients with diabetes and hypertension changes in the albuminuria and the GFR were more frequent and more severe. Furthermore, immediately after the DM2 diagnosis the assessment of protein parameters and the GFR should start because there is a certain percentage of discrepancies between albuminuria and GFR [38-45]. The first is an indicator of kidney

impairment and the GFR - for kidney function. The early diagnosis of DN requires the parallel use of protein parameters and GFR and promoting proteomics urine for new, more perfected biomarkers [43].

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