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Performance of New Biomarkers of Nephropathy in Patients with Type 1 Diabetes Mellitus

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: It is known that laboratorial tests (urinary albumin excretion and glomerular filtration rate), routinely used for nephropathy diagnosis in type 1 diabetes (T1DM), have limitations that justify the evaluation of new renal biomarkers. This study assessed the performance of cystatin C,

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alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT) for nephropathy diagnosis in T1DM patients. The reduction of economic cost and increase in sensibility and specificity from correct biochemical diagnosis of diabetic nephropathy is an important objective of this work.
Methods: Cystatin C, AP and GGT were determined in plasma and urine of healthy individuals (N=35) and T1DM patients with (N=45) and without nephropathy (N=80).
Results: The plasma levels of cystatin C, AP and GGT, as well as urinary levels of cystatin C and AP were able to differentiate diabetic patients with and without nephropathy. Plasma cystatin C better followed the progression of albuminuria. Cystatin C and AP discriminated the onset of nephropathy in T1DM patients better than creatinine. AP plasma/urine ratio progressively increased from the controls to the diabetic patients without and with nephropathy.
Conclusion: The plasma levels of cystatin C and AP may be useful, with the classical markers of renal function, for nephropathy diagnosis and monitoring in T1DM patients.

Keywords: Diabetic nephropathy; cystatin C; alkaline phosphatase; gamma-glutamyl transferase.

ABBREVIATIONS

ACR: Albumin/creatinine ratio; AP: Alkaline phosphatase; APCR: Alkaline phosphatase/creatinine ratio; AUC: Area under the curve; BMI: Body mass index; CCR: Cystatin C/creatinine ratio; CKD: Chronic kidney disease; DM: Diabetes mellitus; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; GCR: Gamma-glutamyl transferase/creatinine ratio; GFR: Glomerular filtration rate; GGT: Gamma-glutamyl transferase; UAE: Urinary albumin excretion; UCR: Urea/creatinine ratio.

1. INTRODUCTION

Diabetes mellitus (DM) often presents chronic complications, which represent a major cause of incapacity, reducing the quality of life and contributing to premature death [1,2]. Among these complications, diabetic nephropathy stands out for both prevalence and morbidity, as well as mortality levels and medical costs associated with the treatment. Diabetic nephropathy affects approximately 35% of patients with type 1 diabetes mellitus (T1DM) and is the leading cause of chronic kidney disease (CKD) in many countries [1,3,4,5]. This condition is characterized by increased urinary albumin excretion (UAE) accompanied by a progressive decline in renal function; thus the importance of diagnosing and monitoring it, according to the determination of UAE and glomerular filtration rate (GFR), which is considered the best overall index of kidney function [5,6,7].

Considering the impact of diabetic nephropathy in public health, both in social and economic terms, it is imperative to diagnosis and monitoring this condition in order to adopt protective and therapeutic measures to prevent or delay its progression. However, laboratorial tests traditionally used in nephropathy in diabetic patients' diagnosis and monitoring, as UAE and GFR, have limitations that compromise their sensibility, specificity and prognostic value. Therefore, it is essential to establish the performance of new biomarkers for nephropathy in diabetic patients in order to overcome the inherent limitations of routine laboratorial tests or, at least, supplement them [8,9,10].

From this perspective, plasma and urine biomarkers have been identified for diagnosis and monitoring, including glomerular and tubular damage markers. The tubular injury biomarkers are essentially plasma low molecular weight proteins such as cystatin C and urinary enzymes secreted by the tubular cells, alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT) [8,10,11,12].

Urinary cystatin C has been considered a potential endogenous GFR biomarker and a tubular injury marker [13-16]. In the presence of glomerular injury, urinary cystatin C may increase due to competitive inhibition of the reabsorption in the renal tubules, which is caused by the concomitant increase of the other low molecular weight proteins [8,12,16,17]. Moreover, the renal tissue is the main source of urinary enzymes excreted and the determination of these enzymes is considered a sensitive and non invasive method for evaluating the tubular cells integrity. The AP (EC 3.1.3.1) and GGT (EC 2.3.2.2) are present in the epithelial cells brush border of the proximal tubules, thus their elevation in urine has been associated with tubular cell injury [10,18]. This study aimed to evaluate the performance of cystatin C, alkaline

phosphatase and GGT levels for nephropathy diagnosis in T1DM patients. The main objectives of this study is to make a cost economic reduction in laboratorial diagnosis of diabetic nephropaty and increase the sensibility and specificity in precoce diagnosis of alterations of renal function in diabetics patients.

2. MATERIALS AND METHODS

2.1 Patients

This study was approved by the local Ethics Committee (CAAE – 0392.0.203.000-11) and all the participants signed a consent form. This study is in accordance to Helsink Declaration.

A total of 160 enrolled participants were distributed into case and control groups. The case group consisted of 125 adults aged 18 to 60 years old, with clinical and laboratory diagnosis of T1DM, with or without diabetic nephropathy, selected in Clinic Hospital, Federal University of Minas Gerais and in Santa Casa de Misericordia of Belo Horizonte/Brazil, from November/2011 to September/2012. The diagnosis of diabetes was performed according to the criteria of ADA [1] and diagnosis of nephropathy according to the guidelines of NKF-KDOQI [7], which considers ACR, GFR estimated by CKD-EPI_{cre} 2009, time of diabetes diagnosis and presence or not of diabetic retinopathy. Pregnant women, cancer patients, alcoholics, patients with liver disease, infectious process in progress, patients on dialysis and kidney transplanted patients were excluded from this group. The control group included 35 healthy individuals aged 18 to 60 without DM, hypertension and kidney disease. Clinical data of the patients were obtained from their medical records and of controls by an interview.

2.2 Biological Samples

Venous blood samples were collected into sodium citrate and centrifuged at 3000 rpm for 15 minutes at 4℃ to obtain the plasma, which was aliquoted and stored at -70℃ until analysis. Urine samples were obtained after retention of 4 hours and were stored at -70℃ until analysis, when they were thawed to room temperature and centrifuged at 2000 x g for 10 minutes at 4℃ to obtain the supernatant.

2.3 Biochemical Determinations

Biochemical determinations were performed in duplicate in the automatic analyzer LabMax 240 using Labtest® kits. Cystatin C plasma and urine levels were determined by immunoturbidimetric, creatinine levels were determined by enzyme-Trinder method traceable from the National Institute of Standards and Technology (NIST) and urea (plasma and urine) by UV enzymatic test (urease). AP and GGT plasma and urine activity were determined by the kinetic method (Bowers and Mc Comb modified and Szasz modified, respectively). Albumin plasma levels were determined by the colorimetric method (Bromocresol green) and urine levels by the immunoturbidimetric method. The urinary levels of each biomarker were corrected by creatinine urinary levels. Plasma/urine ratio of biomarkers was also calculated.

2.4 Statistical Analysis

Statistical analysis was performed using Stata software-version 11.0. The normality of continuous variables was verified by the Shapiro-Wilk test. The variables with parametric distribution were expressed as mean and standard deviation and compared by Student's t test or ANOVA. Variables with non-parametric distribution were expressed as median and interquartile range and compared by Mann-Whitney or Kruskal-Wallis. Categorical variables were presented as absolute and relative frequencies and compared by chi-square test of Pearson or Fisher's exact test. The investigation of the correlation was performed by Spearman correlation coefficient. The diagnostic performance of the biomarkers was evaluated by ROC curve and the interpretation of the area under the curve (AUC) based on the classification proposed by Swets [19]. A two-tailed $P<0.05$ value was considered statistically significant.

3. RESULTS

3.1 Clinical and Laboratory Profile of the Case and Control Groups

The clinical and laboratory data are shown in Table 1. Three groups were tested: The control group, the diabetic group without nephropathy, and the diabetic group with nephropathy. There was no significant difference among the three groups with respect to age, BMI and sex.

The time of diabetes diagnosis, frequency of hypertension and dyslipidemia were higher in the diabetic group with nephropathy than in the diabetic group without nephropathy. Cystatin C, AP and urea plasma levels were significantly higher in T1DM patients with and without

nephropathy than in the controls, and they were higher in T1DM patients with nephropathy than in those without nephropathy. The levels of creatinine and GGT were higher in T1DM patients with nephropathy than in controls or patients without nephropathy. Albumin levels, in turn, showed no significant difference among the groups.

3.2 Clinical and Laboratory Profile of Diabetics as Albuminuria

The clinical and laboratory data of DM1 patients according to the degree of albuminuria are shown in Table 2. Three groups – a group with normoalbuminuria, one with microalbuminuria and a group with macroalbuminuria – were studied. The time of diagnosis, BMI and the percentage of male and female patients did not differ among the groups, whereas age was significantly higher in the group with microalbuminuria than in those with normoalbuminuria and with macroalbuminuria.

The frequency of hypertension was significantly higher in the groups with microalbuminuria and macroalbuminuria than in the group with normoalbuminuria. For dyslipidemia, the frequency was only higher in the group with macroalbuminuria compared to that with normoalbuminuria.

Plasma albumin levels were significantly lower in the group with macroalbuminuria than in those with normoalbuminuria and microalbuminuria. The AP was significantly higher in the group with macroalbuminuria than in those with normoalbuminuria ($P<0.001$) and microalbuminuria ($P= 0.004$). Cystatin C and creatinine plasma levels were higher in the groups with microalbuminuria and macroalbuminuria than in the group with normoalbuminuria, and were also higher in the group with macroalbuminuria than in the microalbuminuria group. GGT and urea plasma levels were higher in the groups with microalbuminuria and macroalbuminuria than in that with normoalbuminuria.

Urinary ACR was higher in the groups with microalbuminuria and macroalbuminuria than in the group with normoalbuminuria and was also higher in the group with macroalbuminuria than in that with microalbuminuria ($P<0.001$). CCR was higher in the group with macroalbuminuria than in those with microalbuminuria and normoalbuminuria. APCR, GCR and UCR showed no significant difference between the groups.

The albumin plasma/urine ratio was lower in the group with microalbuminuria than in that with normoalbuminuria, and it was also lower in the group with macroalbuminuria than in those groups with microalbuminuria and normoalbuminuria. The cystatin C plasma/urine ratio was lower in the group with macroalbuminuria than in those with normoalbuminuria and microalbuminuria. The creatinine and urea plasma/urine ratios were higher in the groups with microalbuminuria and macroalbuminuria than in the group with normoalbuminuria. The AP and GGT plasma/urine ratios showed no significant difference among the groups.

3.3 Variation of Plasma Biomarkers and Clinical Characteristics

Plasma levels of biomarkers in diabetic patients were assessed according to the clinical variables. Cystatin C, creatinine and urea levels were higher in the age groups between 30 – 44 years old and 45 – 60 years old than in that 18 – 29 years old. The GGT levels were higher in the group aged 45 – 60 years old than in those between 18 – 29 years old and between 30 – 44 years old. Albumin and AP levels showed no significant difference between the age categories. Albumin, creatinine and AP values were higher in males, while cystatin C, GGT and urea showed no difference between men and women.

For the time of diabetes diagnosis, only cystatin C and creatinine levels were higher in the group with a diagnosis time over than 20 years compared to the groups with diagnosis time between 11 – 20 years and less than 10 years. Concerning hypertension and dyslipidemia, cystatin C, creatinine, urea and GGT levels were higher in the group with hypertension and were also higher in the group with dyslipidemia. There was no difference when the biomarkers were evaluated with regard to BMI and smoking.

3.4 Correlations between Biomarkers

There was a strongly positive correlation between cystatin C and creatinine. The cystatin C showed moderate positive correlation with urea, AP, GGT and ACR. AP showed a positive moderate correlation with cystatin C, urea, GGT and ACR. GGT showed moderate positive

correlation with cystatin C, creatinine, urea and AP.

3.5 Performance of Biomarkers for Nephropathy Diagnosis in Diabetic Patients

The AUC and the corresponding values of specificity, sensibility and best cut-off of each biomarker in the plasma and urine are shown in Table 3. In plasma (Fig. 1), cystatin C, urea, creatinine, AP and GGT exhibited moderate accuracy for nephropathy diagnosis, whereas the albumin was unable to discriminate the T1DM patients with and without nephropathy. Concerning AUC, it was found that cystatin C, urea and creatinine showed similar performances, and AP and GGT were equivalent to each other. Cystatin C presented the highest values of specificity and sensibility to the biomarkers in plasma.

The ACR showed high accuracy for the nephropathy diagnosis, the CCR showed moderate accuracy in urine (Fig. 2), the APCR and the GCR showed low accuracy and the UCR was unable to discriminate the T1DM patients with and without nephropathy. Regarding the AUC, ACR showed better performance to the other biomarkers in urine; however, its performance was similar to that of cystatin C in plasma. The CCR presented equivalent performance to the APCR and the APCR was equivalent to the GCR. As to specificity and sensibility, both ACR and CCR showed 100% specificity, but the sensibility of ACR was almost three times higher than the CCR. The APCR and GCR also showed high specificity (above 90%), but low sensibility (below 20%).

The plasma/urine ratios (Fig. 3) of urea and creatinine showed moderate accuracy for nephropathy diagnosis, the ratios of AP and GGT showed low accuracy and the ratios of cystatin C and albumin were unable to discriminate DM1 patients with and without nephropathy. According to the AUC, the ratio plasma/urine from urea presented the best performance followed by the same ratio from creatinine. The ratios of AP and GGT were equivalent to each other. For specificity and sensibility, the ratio plasma/urine from urea shown the best combination of values.

4. DISCUSSION

Our data suggest that higher time from diabetes diagnosis presented in T1DM patients with nephropathy compared to T1DM patients without nephropathy could be justified by the fact that the incidence of nephropathy increases as the time of diabetes evolution [20]. The higher frequency of hypertension and dyslipidemia in T1DM patients with nephropathy can be associated with the fact that these comorbidities are risk factors for the nephropathy development [9].

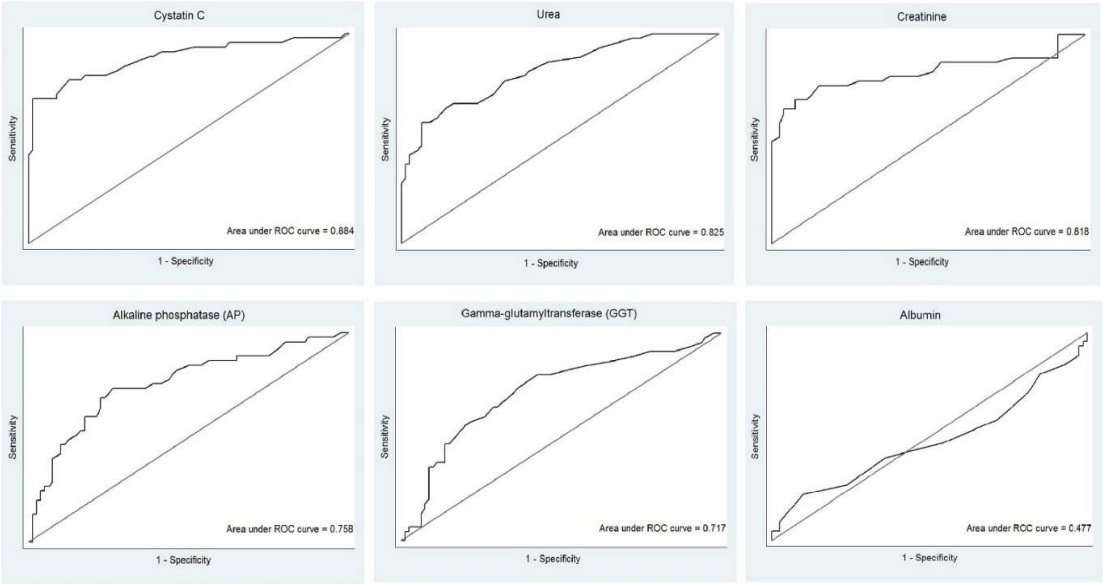

Fig. 1. Performance of biomarkers in plasma for the diagnosis of DN

Table 1. Clinical and laboratory data of the case and control groups

Clinical data	Controls	Diabetics		p
		Without DN	With DN	
Subjects – n℅(%)	35	80 (64)	45 (36)	---
Median Age (years)	30 (26 – 37)	32 (25 – 38)	34 (28 – 44)	0.279
Gender – M/F(%)	13/22 (37/63)	29/51 (36/64)	16/29 (36/64)	0.938
Median BMI (Kg/m^2)	24 (22 – 27)	24 (22 – 26)	23 (21 – 26)	0.380
DM diagnosis time (years)	---	18±8	22±7	0.003*
Hypertension – n℅(%)	---	43 (54)	40 (89)	<0.001*
Dyslipidemia – n℅(%)	---	23 (29)	25 (56)	0.003*
Biomarkers in plasma				
Albumin(g/dL)	3.9 (3.8 – 4.0)	3.9 (3.7 – 4.0)	3.8 (3.6 – 4.1)	0.809
Cystatin C (mg/L)	0.66 (0.49 – 0.80)	0.74 (0.60 – 0.85)^a	1.32 (0.96 – 1.81)^{bc}	p^a =0.031* p^b<0.001* p^c <0.001*
Creatinine (mg/dL)	0.67 (0.60 – 0.73)	0.69 (0.61 – 0.77)	1.21 (0.84 – 1.61)^{bc}	p^b<0.001* p^c <0.001*
AP (U/L)	45 (39 – 57)	56 (46 – 73)^a	83 (64 – 95)^{bc}	p^a < 0.001* p^b<0.001* p^c <0.001*
GGT (U/L)	15 (9 – 28)	14 (11 – 21)	25 (16 – 43)^{bc}	p^b0.006* p^c <0.001*
Urea (mg/dL)	21 (18 – 26)	25 (22 – 30)^a	42 (29 – 58)^{bc}	p^a =0.002* p^b<0.001* p^c <0.001*
Biomarkers in urine				
ACR (mg/g)	2.6 (1.2 – 4.0)	4.2 (2.6 – 10.7)^a	395.6 (62.4 -1527.7)^{bc}	p^a <0.001* p^b<0.001* p^c <0.001*
CCR (mg/g)	0.08 (0.03 – 0.10)	0.06 (0.03 – 0.09)	0.09 (0.06 – 0.52)^{bc}	p^b0.033* p^c <0.001*
APCR (U/g)	1.0 (0 – 1.9)	1.5 (0.1 – 3.8)	3.0 (1.0 – 4.9)^{bc}	p^b=0.001* p^c =0.031*
GCR (U/g)	0.7 (0.2 – 1.7)	1.0 (0.3 – 11.4)	1.3 (0.5 – 16.9)^b	p^b=0.005*

Clinical data	Controls	Diabetics		p
		Without DN	With DN	
UCR (mg/g)	12700 (8437 – 13897)	14652 (11231 – 17825)[a]	13173 (10951 – 16415)	p^a =0.003*
Plasma/urine ratio				
Albumin	8298 (6000 – 13226)	7045 (3229 – 18500)	130 (34 – 1007)[bc]	p^b<0.001* p^c <0.001*
Cystatin C	6.0 (2.8 – 17.4)	11.6 (5.1 – 37.2)[a]	9.5 (4.0 – 28.3)	p^a =0.012*
Creatinine	0.004 (0.003 – 0.007)	0.006 (0.004 – 0.010)[a]	0.013 (0.008-0.028)[bc]	p^a =0.009* p^b<0.001* p^c <0.001*
AP	21.0 (13.5 – 29.8)	25.7 (15.7 – 54.0)	34.7 (18.8 – 58.4)	0.187
GGT	11.9 (5.0 – 25.8)	13.2 (1.3 – 30.2)	12.7 (1.5 – 33.3)	0.698
Urea	0.012 (0.009 – 0.016)	0.016 (0.012 – 0.026)[a]	0.034 (0.025 – 0.069)[bc]	p^a <0.001* p^b<0.001* p^c <0.001*

**Significant difference; DN: diabetic nephropathy; AP: alkaline phosphatase; GGT: gamma-glutamyl transferase; ACR: albumin/creatinine ratio; CCR: cystatin C/creatinine ratio; APCR: AP/creatinine ratio; GCR: GGT/creatinine ratio; UCR: urea/creatinine ratio;*

GFR: glomerular filtration rate. Categorical variables were expressed as absolute and relative frequency and compared by chi-square or Fisher's exact test. Continuous variable with parametric distribution were expressed as mean and standard deviation and compared by Student's t test. Continuous variables with non-parametric distribution were expressed as median and interquartile range and compared by Kruskal-Wallis or Mann-Whitney

[a]p<0.05 for diabetics without DN x controls

[b] p<0.05 for diabetics with DN x controls

[c]p<0.05 for diabetics with DN x diabetics without DN

Table 2. Clinical and laboratory data of diabetics as albuminuria

Clinical data	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p
Subjects – n℅(%)	84 (67)	16 (13)	25 (20)	---
Age (years)	32 (26 – 40)	38 (32 – 44)[a]	30 (23 – 38)[c]	p^a =0.031* p^c =0.024*
Gender – M/F(%)	31/53 (37/63)	4/12 (25/75)	10/15 (40/60)	0.613
BMI (Kg/m^2)	24 (22 – 26)	24 (21 – 27)	22 (20 – 24)	0.254
DM diagnosis time (years)	18 ± 8	23±7	22±7	0.059
Hypertension – n℅(%)	46 (55)	15 (94) [a]	22 (88)[b]	p^a =0.004* p^b=0.002*
Dyslipidemia – n℅(%)	26 (31)	8 (50)	14 (56) [b]	p^b=0.002*
Biomarkers in plasma				
Albumin (g/dL)	3.9 (3.7 – 4.0)	4.0 (3.8 – 4.4)	3.6 (3.4 – 4.0)[bc]	p^b=0.005* p^c =0.001*
Cystatin C (mg/L)	0.74 (0.61 – 0.87)	1.02 (0.87 – 1.48)[a]	1.59 (1.07 – 2.24)[bc]	p^a <0.001* p^b<0.001* p^c <0.001*
Creatinine (mg/dL)	0.69 (0.61 – 0.79)	0.91 (0.68 – 1.24)[a]	1.39 (0.85 – 1.80)[bc]	p^a =0.010* p^b<0.001* p^c =0.049*
AP (U/L)	58 (48 – 74)	68 (57 – 84)	89 (79 – 128)[bc]	p^b<0.001* p^c =0.004*
GGT (U/L)	14 (11 – 21)	30 (14 – 42)[a]	25 (18 – 50)[b]	p^a =0.029* p^b<0.001*
Urea (mg/dL)	25 (22 – 31)	38 (29 – 50)[a]	42 (29 – 59)[b]	p^a <0.001* p^b<0.001*
Biomarkers in urine				
ACR (mg/g)	4.3 (2.6 – 10.9)	63.5 (42.0 – 133.4)[a]	1266.2 (691.3 – 2476.6)[bc]	p^a <0.001* p^b<0.001* p^c <0.001*
CCR (mg/g)	0.06 (0.03 – 0.09)	0.06 (0.04 – 0.09)	0.25 (0.07 – 1.09)[bc]	p^b<0.001* p^c <0.001*
APCR (U/g)	1.5 (0.1 – 3.8)	3.0 (1.15 – 4.85)	3.0 (1.3 – 4.9)	0.074
GCR (U/g)	1.0 (0.3 – 11.0)	5.8 (0.8 – 18.2)	1.1 (0.4 – 21.5)	0.195
UCR (mg/g)	14652 (11178 – 17522)	13752 (10952– 19342)	12771 (10420 – 15569)	0.363

Clinical data	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p
Plasma/urine ratio				
Albumin	7045 (2847 – 18500)	1035 (408 – 1284)[a]	34 (21 – 63)[bc]	p^a <0.001* p^b<0.001* p^c <0.001*
Cystatin C	11.8 (5.0 – 37.2)	26.2 (9.2 – 52.4)	4.6 (3.4 – 11.2)[bc]	p^b=0.004* p^c =0.003*
Creatinine	0.006 (0.004 – 0.010)	0.011 (0.007 – 0.016)[a]	0.017 (0.009 – 0.049)[b]	p^a =0.008* p^b<0.001*
AP	25.5 (14.7 – 54.6)	36.0 (18.7 – 42.4)	33.5 (23.4 – 76.0)	0.312
GGT	13.2 (1.3 – 29.9)	3.1 (1.1 – 51.4)	18.8 (1.9 – 33.3)	0.571
Urea	0.018 (0.013 – 0.027)	0.032(0.018–0.046)[a]	0.047 (0.029 – 0.116)[b]	p^a =0.001* p^b<0.001*

**Significant difference; AP: alkaline phosphatase;*
GGT: gamma-glutamyl transferase; ACR: albumin/creatinine ratio;
CCR: cystatin C/creatinine ratio; APCR: AP/creatinine ratio; GCR: GGT/creatinine ratio;
UCR: urea/creatinine ratio;
GFR: glomerular filtration rate. Categorical variables were expressed as absolute frequency and relative and compared by Fisher's exact test
Continuous variable with parametric distribution were expressed as mean and standard deviation and compared by Student's t test
Continuous variables with non-parametric distribution were expressed as median and interquartile range and compared by Kruskal-Wallis or Mann-Whitney.
[a]p<0.05 for diabetic patients with microalbuminuria x diabetic patients with normoalbuminuria
[b] p<0.05 for diabetic patients with macroalbuminuria x diabetic patients with normoalbuminuria
[c]p<0.05 for diabetic patients with macroalbuminuria x diabetic patients with microalbuminuria

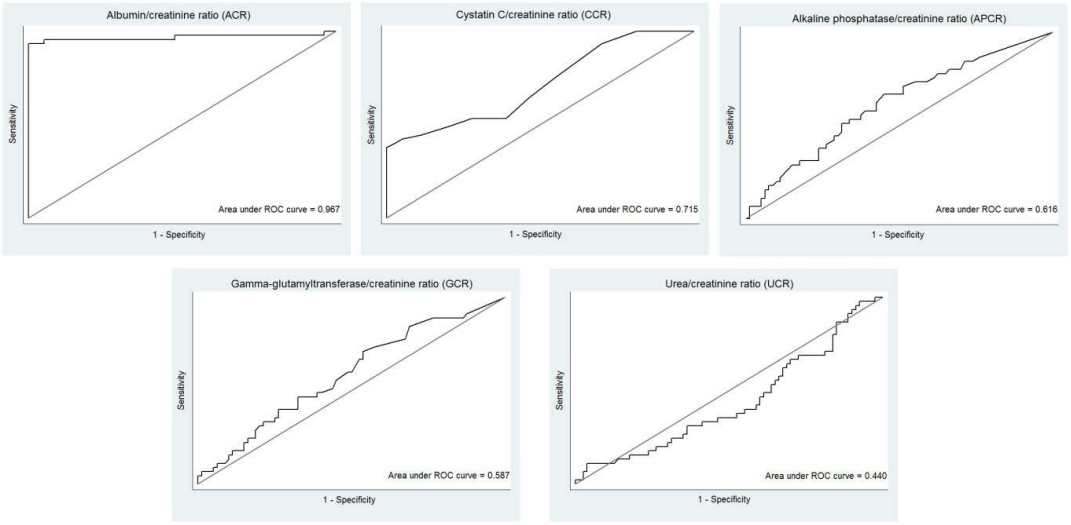

Fig. 2. Performance of biomarkers in urine for the diagnosis of DN

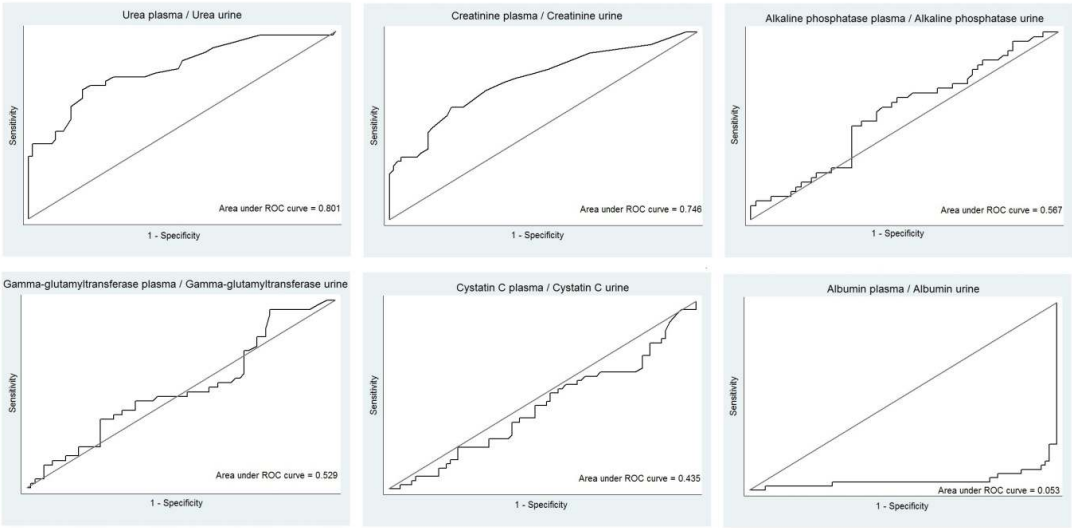

Fig. 3. Performance of plasma/urine ratios for the diagnosis of DN

Our data showed that the highest values of cystatin C, creatinine and urea plasma levels in T1DM patients with nephropathy could be correlated to the decrease of GFR, which normally occurs with the progression of nephropathy. Similar to our findings, Wang et al. [21] also observed higher values of cystatin C in patients with type 2 diabetes mellitus (T2DM) and nephropathy compared toT2 DM patients without nephropathy.

T1DM patients with and without nephropathy had also higher AP plasma levels than controls, which can be explained by the association between high AP levels and metabolic diseases such as DM [22]. The highest GGT level in T1DM patients with nephropathy was expected, since GGT has been considered a predictor of microalbuminuria and CKD in DM patients [23].

Unlike creatinine, cystatin C, AP and urea were higher in the T1DM patients without nephropathy than in controls, which suggest that these three biomarkers rise earlier in DM patients than creatinine. This indicates that cystatin C, AP and urea discriminate better the onset of nephropathy inT1DM patients than creatinine.

Table 3. Performance of biomarkers in the diagnosis of DN

	AUC	Cutoff	Specificity (%)	Sensitivity (%)
Biomarkers in plasma				
Cystatin C (mg/L)	0.884	1,05	99	69
Urea (mg/dL)	0.825[a]	40	94	58
Creatinine (mg/dL)	0.818[ab]	0,93	96	64
AP (U/L)	0.758[bc]	79	82	60
GGT (U/L)	0.717[cd]	24	80	56
Albumin (g/dL)	0.477	4,2	90	22
Biomarkers in urine				
ACR (mg/g)	0.967[a]	28,2	100	93
CCR (mg/g)	0.715[bcde]	0,15	100	38
APCR (U/g)	0.616[ef]	5,8	92	18
GCR (U/g)	0.587[eg]	52	99	7
UCR (mg/g)	0.440	21712	96	11
Plasma/urine ratio				
Urea	0.801[bcdef]	0,03	83	69
Creatinine	0.746[bcdef]	0,025	98	31
AP	0.567[fgh]	382,2	98	10
GGT	0.529[ghi]	105,4	94	12
Cystatin C	0.435	870	100	0
Albumin	0.053	390000	100	0

DN: diabetic nephropathy; AUC: are under the curve; AP: alkaline phosphatase; GGT: gamma-glutamyl transferase; ACR: albumin/creatinine ratio; CCR: cystatin C/creatinine ratio; APCR: AP/creatinine ratio; GCR: GGT/creatinine ratio; UCR: urea/creatinine ratio

ap>0.05 between biomarker x cystatin C
bp>0.05 between biomarker x urea
cp>0.05 between biomarker x creatinine.
dp>0.05 between biomarker x AP
ep>0.05 between biomarker x GGT
fp>0.05 between biomarker x CCR
gp>0.05 between biomarker x APCR
hp>0.05 between biomarker x GCR
ip>0.05 between biomarker x plasma/urine ratio AP

In urine, the largest ACR values observed in T1DM patients compared to controls and in patients with nephropathy compared to those without nephropathy could be justified by the fact that chronic hyperglycemia compromises the glomerular basement membrane as it determines selectivity and size, favoring the increase of urinary albumin excretion [24].

Higher CCR, APCR and GCR in DM1 patients with nephropathy should be associated with tubular injury. It is known that the tubular lesion commits reabsorption of cystatin C by the proximal tubules, which favors the increase of its urinary excretion [8,12]. The higher APCR and GCR values may indicate lesion of the tubular cells that secrete such enzymes [10,18].

Regarding plasma/urine ratios, our data shown that urea, creatinine and albumin were more efficient for discriminating healthy subjects and diabetic patients, as well as T1DM patients with and without nephropathy than the new biomarkers evaluated. Although the AP plasma/urine ratio did not show statistical difference among groups, it is important to emphasize that in terms of absolute value the AP ratio progressively increased from the control group to diabetic patients without nephropathy and diabetic patients with nephropathy, which indicates a good ability of this enzyme in the evaluation of diabetic patients. Perhaps, if evaluated diabetic patients with metabolic and hemodynamic profile more homogeneous and/or other statistical tests were applied, the AP ratio could be able to discriminate healthy individuals from patients with diabetes and also diabetic patients with and without nephropathy.

For albuminuria, it was found that hypertension was more frequent in patients with microalbuminuria and macroalbuminuria than in patients with normoalbuminuria, which

corroborates the statement that hypertension in T1DM may also be a consequence of nephropathy progression [9]. Regarding to dyslipidemia, which was more frequent in patients with macroalbuminuria compared to patients with normoalbuminuria, our results agree with the previous studies involving patients withT2DM [12,25].

In plasma, the results showed that cystatin C and creatinine were the biomarkers that best tracked the progression of albuminuria, since they present significant differences among all albuminuria levels, unlike the other markers.

In urine and plasma/urine ratio, albumin best accompanied the progression of albuminuria, showing a significant difference between the three stages. It was able to detect the evolution of normoalbuminuria to microalbuminuria, which confirms its value in early diagnosis of nephropathy. Cystatin C seems to be a later biomarker, since no difference between the stages of normoalbuminuria and microalbuminuria was found. AP and the GGT did not follow the progression of albuminuria in any of the stages.

Concerning the variation of plasma biomarkers according to the clinical characteristics, increased cystatin C, creatinine and urea with the progression of age can be explained by the decline in renal function that occurs with aging itself. Creatinine and cystatin C were also higher in diabetic patients with diagnosis time more than 20 years, which can be explained by the decline of renal function which often occurs in parallel to the evolution of DM. This result shows that cystatin C and creatinine were more effective for monitoring renal function in DM1 patients than the other biomarkers.

For gender, there was no significant difference in cystatin C levels, unlike creatinine, that appeared higher in men, possibly under the influence of muscle mass [14]. This result makes the use of cystatin C interesting due to the possibility of adopting a single reference value. Similar to our data, Woo et al. [26] also found no difference in cystatin C levels between men and women with nephropathy.

The highest values of cystatin C, creatinine and urea in hypertensive patients corroborate the statement that hypertension contributes to the development of diabetic nephropathy [9]. These three biomarkers also showed higher in patients with dyslipidemia. Previous studies have reported an association between higher levels of triglycerides in correlation with higher levels of cystatin C and creatinine, as well as an association between higher levels of HDL and lower levels of cystatin C [27]. The GGT was also higher in hypertensive and dyslipidemic patients, which is justified by the association of this enzyme with high blood pressure and obesity [23]. The AP and GGT also increase due to microangiopathy. Because of this, these markers generally increase at various disease such as nephropathy, liver disease and malignancies [28 a and b].

The new biomarkers did not differ according to the BMI, which favors the use of them in clinical practice, given the significant number of subjects with overweight/obesity in diabetic population. Another factor favoring the use of new biomarkers is that they have not presented differences in smoking.

New biomarkers cystatin C, AP and GGT, besides having moderate correlation between them, showed moderate to strong correlations with the traditional markers of kidney function (urea and creatinine), as well as the ACR, which is recommended for the diagnosis of nephropathy. Similar to our findings, Bulum et al. [29] evaluated patients with T1DM and found significant correlation between GGT and creatinine. In patients with T2DM, Jeon et al. [25] also found a significant correlation of cystatin C and creatinine and ACR and Carvalho et al. [18] reported a moderate correlation between AP and ACR.

For diabetic nephropathy diagnosis, the good performance presented by the plasma cystatin C, AP and GGT can be explained, at least in part, because they are correlated with the stage of renal function, which is evidenced by their correlation with creatinine, urea and ACR. The high accuracy presented by the ACR is justified by the fact that the classification of patients for the presence of nephropathy was conducted taking into account the GFR and the ACR.

In plasma, the biomarker of choice would be cystatin C, because despite having equivalent performance to urea and creatinine, it showed higher specificity and sensitivity. In urine and for plasma/urine ratio, biomarkers of choice would be ACR and the urea ratio, respectively, as they exhibited superior performances to other biomarkers and best specificity and sensitivity combinations.

Generally, for the diagnosis of nephropathy in diabetic patient, plasma cystatin C was the biomarker that is closer to the ACR, showing equivalent performance to it and a very close specificity. However, the ACR presented a greater sensitivity than cystatin C, which makes it the biomarker of choice among all the others.

5. CONCLUSION

In conclusion, plasma was the best sample for assessing the cystatin C, AP and GGT in DM1 patients, since their plasma levels were able to differentiate diabetic patients with and without nephropathy. It should be highlighted that cystatin C also distinguished healthy individuals from diabetic patients, as well as diabetic patients with and without nephropathy. Furthermore, it followed the progression of albuminuria and showed good performance for nephropathy diagnosis. Cystatin C and AP discriminated the onset of nephropathy in DM1 patients better than creatinine. Besides, the AP plasma/urine ratio progressively increased from the controls to the diabetic patients without and with nephropathy, which justifies further studies evaluating AP in DM patients. By the way the most sensibility of cystatin C than creatinine verified in our results and the potential of Alkaline Phosphatase (AP), that lower economic cost is a important evolution from precoce detection of diabetic nephropathy.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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