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THE SIGNIFICANCE OF URINARY MARKERS IN THE EVALUATION OF DIABETIC NEPHROPATHY

ZNAČAJ ODREĐIVANJA URINARNIH MARKERA U PRAĆENJU DIJABETESNE NEFROPATIJE

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Summary: Oxidative stress is considered to be a unifying link between diabetes mellitus (DM) and its complications, including nephropathy (DN). The aim of this study was to determine the parameters of oxidative injury of lipids and proteins as well as the activity of ectoenzymes in the urine of DN patients. The study included 40 individuals: 10 patients with type 2 diabetes mellitus and microalbuminuria (DMT2-MIA), 10 type 2 diabetic patients with macroalbuminuria (DMT2-MAA), 10 patients with type 1 diabetes and microalbuminuria (DMT1-MIA) and 10 age- and sex-matched healthy subjects (control). In the urine we determined TBA reactive substances (TBARS), reactive carbonyl groups (RCG), and the activity of ectoenzymes *N*-acetyl-β-d-glucosaminidase (NAG), plasma cell differentiation antigen (PC-1), aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV). A higher concentration of TBARS in the urine was found in DMT2-MIA and DMT1-MIA, compared to the control group ($p<0.001$ and $P<0.05$). The urine concentration of RCD shows similar results with a significant elevation in the groups with DMT2-MAA and DMT1-MIA, compared to the DMT2-MIA ($p<0.001$) and control group ($p<0.001$). Activities of NAG, APN and DPPIV were significantly higher in the urine of DMT2-MAA, compared to the control ($p<0.01$). The activity of PC-1 was slightly increased in that group, but not significantly. In conclusion, the level of oxidative stress markers and activities of brush border ectoenzymes in the urine may be a useful non-invasive and easily repeatable test in DN.

Keywords: oxidative stress, ectoenzyme, urine, diabetic nephropathy

Kratak sadržaj: Oksidativni stres može se smatrati jedinstvenim faktorom koji povezuje dijabetes melitus (DM) i njegove komplikacije, uključujući nefropatiju (DN). Cilj ovog istraživanja bio je da se u urinu pacijenata sa DN odrede parametri oksidativnog oštećenja lipida i proteina kao i aktivnost ektoenzima. Istraživanjem je obuhvaćeno 40 pacijenata: 10 pacijenata sa dijabetes melitusom tipa 2 i mikroalbuminurijom (DMT2-MIA), 10 dijabetičnih pacijenata tipa 2 sa makroalbuminurijom (DMT2-MAA), 10 pacijenata sa dijabetom tipa 1 i mikroalbuminurijom (DMT1-MIA) i 10 zdravih osoba (kontrola). U urinu je određivana koncentracija TBA reaktivnih supstanci (TBARS), reaktivne karbonilne grupe (RCG) i aktivnost ektoenzima *N*-acetil-β-d-glukozaminidaze (NAG), plazma ćelijski diferencirajući antigen (PC-1), aminopeptidaza N (APN) i dipeptidil peptidaza IV (DPP IV). Veća koncentracija TBARS u urinu nađena je u DMT2-MIA i DMT1-MIA grupi, u odnosu na kontrolu ($p<0,001$ i $p<0,05$). Koncentracija RCD u urinu pokazuje slične vrednosti sa statistički značajnim povećanjem u DMT2-MAA i DMT1-MIA grupi u odnosu na DMT2-MIA ($p<0,001$) i kontrolnu grupu ($p<0,001$). Aktivnost NAG, APN i DPPIV značajno je veća u urinu pacijenata sa DMT2-MAA u odnosu na kontrolu ($p<0,01$). Aktivnost PC-1 pokazuje lagani, ali ne i signifikantan porast u toj grupi pacijenata. Zaključujemo da praćenje markera oksidativnog stresa i aktivnost *brush border* ektoenzima u urinu mogu biti korisni, neinvazivni i lako primenljivi testovi u praćenju DN.

Ključne reči: oksidativni stres, ektoenzimi, urin, dijabetesna nefropatija

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Introduction

Diabetes is recognized as one of the leading causes of morbidity and mortality in the world. By the year 2010, the total number of people with diabetes is estimated to reach 221 million (1). About 40% of types

1 and 2 diabetic patients develop diabetic nephropathy and retinopathy as microangiopathy, in addition to cardiovascular complications in the long-term course of their disease. Diabetic nephropathy is the leading cause of end-stage renal failure, accounting for 35 to 40% of all new cases that require dialysis therapy worldwide (2). Oxidative stress is considered to be a unifying link between diabetes mellitus (DM) and its complications, including nephropathy. Oxidative stress has been defined as a loss of balance between free radical production and the antioxidant systems, with negative effects on carbohydrates, lipids, and proteins. Hyperglycemia is generally accepted as the major cause of diabetic microvascular complications and may play an important role in the development of macrovascular diseases. Elevated glucose causes oxidative stress due to increased production of mitochondrial ROS, nonenzymatic glycation of proteins and glucose autoxidation (3). High glucose induces intracellular ROS in mesangial and tubular epithelial cells and acting as integral glucose signaling molecules in the diabetic kidney (4). Nonenzymatic reactions between sugars and the free amino groups of proteins, lipids, and nucleic acids result in molecular dysfunction through the formation of advanced glycation end products (AGE). They are able to stimulate directly the production of extracellular matrix, inhibit its degradation and disrupt matrix-matrix and matrix-cell interactions, contributing to their profibrotic action (5). Diabetic nephropathy is characterized by the accumulation of extracellular matrix (ECM) protein in the glomerular mesangium and tubulointerstitium. In simple terms, this can be explained as an imbalance between the synthesis and degradation of ECM components, leading to the pathologic accumulation of collagens, fibronectins, and laminins. AGE is able to influence this balance in a variety of ways. Because of their slow turnover, ECM proteins are especially susceptible to AGE modification, resulting in alterations of both structure and function. The formation of inter- and intramolecular cross-links after the glycation of collagen leads to structural alterations, including changes in packing density and surface charge, manifested by increased stiffness, reduced thermal stability, and resistance to proteolytic digestion. They can also impair the binding of heparan sulfate to the extracellular matrix, which results in a loss of anionic sites and thus in an increase in endothelial permeability. Morphologically, the development of diabetic nephropathy is characterized by a progressive thickening of the glomerular basement membrane and an expansion of the mesangial matrix which correlates to the glomerular filtration function. Electron microscopic studies show that the increase of glomerular extracellular matrix correlates well with the extent of microalbuminuria (6). This correlation is found in both type 1 and type 2 diabetic patients. Microalbuminuria appears 10–15 years after hyperglycemia promoting during the next 3–5 years into distinct proteinuria and DN (7). Microalbuminuria can be defined as albumin excretion of 20 to 200 mg/min or 30 to 300 mg/24 h. It can be detected in almost 10–35% patients with insulin dependent and 8–25% insulin independent diabetes mellitus (8).

There is an intensive effort invested in the search for new early specific urine markers of glomerular and tubular injury in DN. Degradation of lipid peroxides generates different compounds, such as malondialdehyde (MDA) which may be transported or simply leak from the organ or tissue of origin into the bloodstream and become excreted in urine. Just one oxidative stress markers in urine are reactive carbonyl derivates (RCD), as a measure of the oxidative modification of proteins. The AGE hypothesis proposes that accelerated chemical modification of proteins by glucose during hyperglycemia contributes to the pathogenesis of diabetic nephropathy (9).

N-acetyl-β-d-glucosaminidase (NAG) is a lysosomal enzyme present in high concentrations in renal proximal tubular cells (10). The molecular weight of 140 kDa does not permit glomerular filtration of NAG, thus, their increased urinary excretion is one of the most sensitive markers of renal proximal tubular cells injury. Urinary NAG excretion has been recommended as a useful marker for the detection of changes in proximal tubular function long before elevations in other markers, such as proteinuria and serum creatinine (11). Plasma cell differentiation antigen (PC-1) is an inhibitor of the insulin receptor tyrosine kinase implicated in the pathogenesis of insulin resistance. It is an ectoenzyme, possessing both alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. Urinary PC-1 was found to be mainly produced by the kidney (12). Aminopeptidase N (EC 3.4.11.2, CD13, APN) is a 150 kDa integral membrane-bound protein which cleaves bioactive oligopeptides with N-terminal preferentially neutral amino acids. APN have the highest activity on the brush border of proximal tubular cells (13), but they are also widely distributed on the membranes of human resting and antigen-stimulated lymphocytes (14). Dipeptidyl peptidase IV (EC 3.4.14.5, CD 26, DPP IV) is a membrane-bound serine protease, present on the brush border of different epithelia, mainly on proximal tubular cells. It is a 110 kDa integral type II glycoprotein that liberates X-Pro and X-Ala dipeptides from the N terminus of different bioactive polypeptides, including hormones, neuropeptides, cytokines and chemokines (15). The current literature indicates a direct association of T cells DPP IV with adenosine deaminase and extracellular matrix components, such as fibronectin and collagen (16, 17).

Therefore, the knowledge of the molecular mechanism of DN pathogenesis may be helpful in developing new therapeutic strategies, as well as in establishing diagnostic markers for its early detection. The main goal of this study was to determine the parameters of oxidative injury of lipids and proteins as well as the activity of ectoenzymes (NAG, PC-1, APN, and DPP IV) in the urine of DN patients.

Material and Methods

The study included 40 individuals: 10 patients with type 2 diabetes mellitus and microalbuminuria (DMT2-MIA), 10 type 2 diabetic patients with macroalbuminuria (DMT2-MAA), 10 patients with type 1 diabetes and microalbuminuria (DMT1-MIA) and 10 age- and sex-matched healthy subjects (control). Microalbuminuria presents a persistant increase in the albumin excretion rate to values of between 30 and 300 mg/24 h, and macroalbuminuria is a value higher than 300 mg/24 h. None of the patients was known to suffer from an acute illness or chronic inflammatory condition at the time of the study. Blood samples were drawn in the fasting state and processed within 1 hour of collection. The first morning urine sample was collected after the centrifugation into a sterile tube and stored at –20 °C until analysis.

Glucose and creatinine levels were measured using an autoanalyzer A25 (»Bio Systems«). Fructosamine was determined in serum with a colorimetric assay based on the ability of ketoamines to reduce nitrobluetetrazolium (NBT) to formazan in an alkaline solution (18).

For the determination of the concentration of thiobarbituric acid-reacting substances (TBARS), urine was combined with 5% butylated hydroxitoluene (BHT) and thiobarbituric acid (TBA) solution. After incubation at 100 °C, the absorbance of samples at 532 nm was measured and calculated using the molar extinction coefficient and expressed as μM/mM creatinine (19). The protein oxidation level was monitored by a spectrophotometric determination of carbonyl content by the method of Levine using 2,4-dinitrophenylhydrazine (DNPH) as a classic carbonyl reagent (20). In brief, protein precipitated with sulphosalicilic acid for urine was centrifuged and incubated in a solution containing DNPH for 50 min at 37 °C. Spectrophotometric measurement of reactive carbonyl derivates (RCD) values was performed and calculated using the extinction coefficient of DNPH-reactive carbonyl derivates at 370 nm and expressed as μM/mM creatinine. Urinary *N*-acetyl-β-d-glucosaminidase (NAG) activity was determined according to the method of Horak et al. (21). The amount of *p*-nitrophenol was measured spectrophotometrically at 405 nm. The activity was represented as nmol of *p*-nitrophenol formed per min. Urinary NAG excretion was expressed as U/mM creatinine to rule out the influence of urinary dilution or concentration. The phosphodiesterase activity of PC-1 was measured by the hydrolysis of thymidine 5′-monophosphate p-nitrophenyl ester. PC-1 activity was determined using 5 mmol/L substrate dissolved in 50 mmol/L TRIS-HCl buffer pH 8.5, containing 130 mmol/L NaCl and 1 mmol/L $MgCl_2$. Incubation was monitored at 37 °C for 3–10 min. under zero-order kinetic conditions. The reaction was stopped with the addition of 1 mol/L NaOH. The formed *p*-nitrophenol was measured on OD of 405 nm (22). The activity of APN was measured in phosphate buffer saline, supplemented with 1 mol/L $MgCl_2$, pH 7.4, using 1.5 mol/L alanine-*p*-nitroanilide as chromogenic substrate. DPP IV activity was determined in 50 mol/L TRIS-HCl, pH 7.8 containing 130 mol/L NaCl and 1 mol/L $MgCl_2$ with 1.5 mol/L gly-pro-*p*-nitroanilide as substrate. Incubation was carried out at 37 °C for 10–30 min under zero-order kinetic conditions. Enzyme reaction was stopped with 0.1 mL of 10% (v/v) trichloracetic acid. The amount of p-nitroanilide formed was measured by the reading on OD of 405 nm (23).

For statistical analysis the Student's t-test was used when appropriate. Data were expressed as mean ± S.D. Comparisons of the enzyme activity between different groups were carried out using the Mann-Whitney U-test; differences were considered significant at $p<0.05$.

Results

The clinical and biochemical data of healthy and diabetic subjects are summarized in *Table I*. Patients with diabetic nephropathy had significantly higher serum concentrations of glucose compared to the control group ($p<0.001$). Diabetic patients with DMT1-MIA had higher levels of glucose than DMT2-MIA ($p<0.05$). Fructosamine levels were increased in all diabetic groups compared to the healthy control subjects ($p<0.001$, and $p<0.01$ for DMT2-MAA group). In addition, a significant difference in the fructosamine level was observed between the diabetic patient groups, DMT2-MAA patients had lower levels compared to the DMT2-MIA ($p<0.05$), and DMT1-MIA compared to the DMT2-MAA ($p<0.05$). Creatinine concentration was higher in both groups of type 2 dia-

Table I Biochemical parameters in the serum of diabetic patients and the control group.

	Control group (n=10)	Diabetic groups (n=30)		
		DMT2-MIA	DMT2-MAA	DMT1-MIA
Female/male	5/5	8/2	4/6	4/6
Glucose (mmol/L)	5.02 ± 0.50	10.11 ± 1.72[b]	12.61 ± 3.97[b]	14.36 ± 6.32[b,c]
Creatinine (μmol/L)	69.11 ± 6.79	91.50 ± 18.76[a]	123.00 ± 37.48[b,c]	81.55 ± 17.11
Fructosamine (mmol/L)	1.29 ± 0.32	2.42 ± 0.48[b]	1.89 ± 0.29[a,c]	2.63 ± 0.67[b,d]

Values are means ± S.D. [a]$P < 0.01$ vs. control; [b]$P < 0.001$ vs. control; [c]$P < 0.05$, vs. DMT2- MIA; [d]$P < 0.05$ vs. DMT2-MAA.

betic patients compared to healthy subjects. No differences have been found between the control and DMT1-MIA groups.

A higher concentration of TBARS in urine (*Figure 1*) was found in DMT2-MIA and DMT1-MIA compared to the control group ($p<0.001$ and $p<0.05$).

The urine concentration of RCD showed similar results with a significant elevation in the groups with DMT2-MAA and DMT1-MIA compared to the DMT2-MIA ($p<0.001$) and control group ($p<0.001$) (*Figure 2*)).

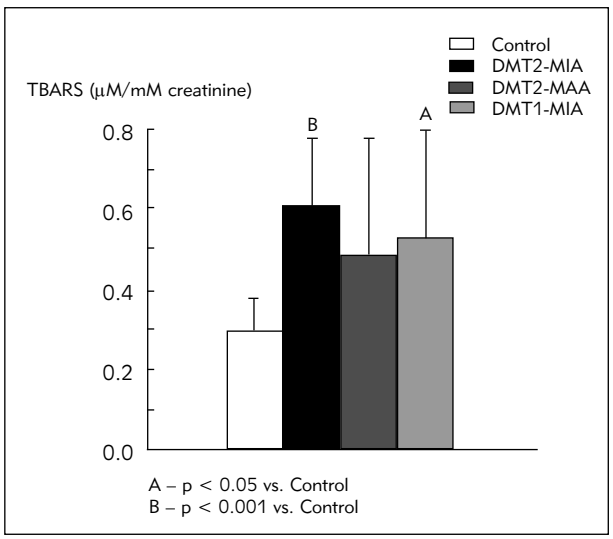

Figure 1 Thiobarbituric acid reactive derivates in the urine of patients with diabetic nephropathy and control subjects.

The activities of NAG, APN and DPPIV were significantly higher in the urine of DMT2-MAA compared to the controls ($p<0.01$). The activity of PC-1 was slightly increased in that group, but not significantly (*Figure 3*).

Discussion

Diabetes is a serious public health problem considering its frequency, complications and the growing burden of costs for the healthcare systems (24). Multifactorial complication and its long-term consequences involving chronic renal insufficiency and increased rate of cardiovascular death are the main characteristics of DN. In both type 1 and type 2 diabetes, diabetic complications in target organs arise from chronic elevations of glucose. Oxidative stress is apparent during the stage of glucose intolerance, long before DM becomes clinically visible. High glucose induces intracellular reactive oxygen species (ROS) in the mesangial and tubular epithelial cells. In addition to their ability to directly inflict macromolecular damage, ROS function as signaling molecules activating a number of cellular stress-sensitive pathways, ultimately responsible for the late complications of diabetes.

In this study, a higher level of fructosamine in the diabetic patients was found, indicating poor glycemic control. Moreover, patients with DMT2-MAA have increased creatinine concentrations compared to the other two groups of diabetic patients and the control group (*Table 1*). Besides the classical clinical chemical parameters for the evaluation of renal function, the measurement of urinary albumin excretion is now wide-

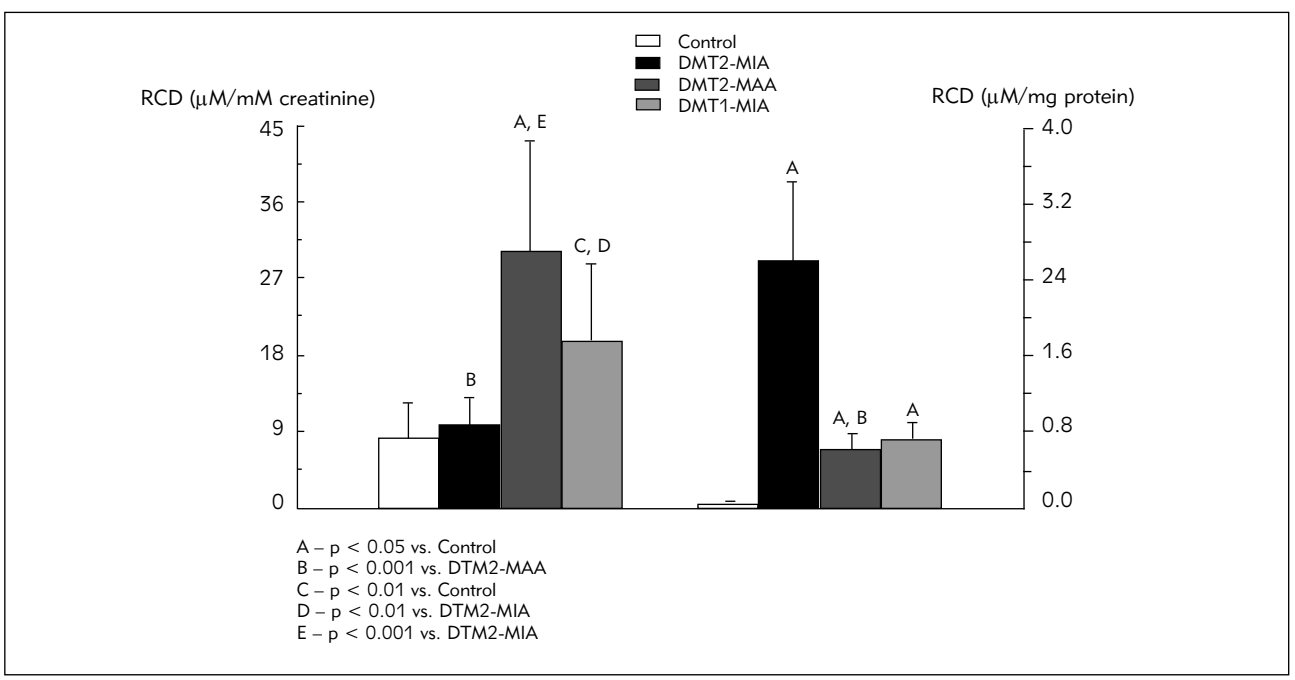

Figure 2 Reactive carbonyl groups in the urine of patients with diabetic nephropathy and control subjects.

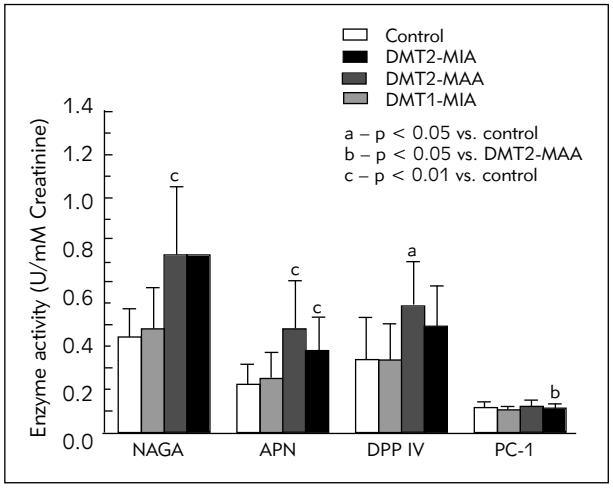

Figure 3 Activities of proximal tubule ectoenzymes in the urine of patients with DN.

ly used for detection of developing DN. Since diabetes causes glomerular and tubular changes, tubular marker proteins may be used to detect early renal damage. Microalbuminuria and subsequent progression to proteinuria and nephropathy is associated with increased oxidative stress, increased inflammatory cytokines production and elevated cardiovascular (CVD) risk (25).

At the same time, in this study a significantly higher level of TBARS in the urine of type 1 and type 2 diabetic patients with microalbuminuria has been demonstrated. Some compounds generated as the product of lipid peroxides degradation (alkanes, alkenals, and hydroxyalkanes) could be transported into the bloodstream, becoming excreted in urine (26). A recent study of Chang (27) has shown that type 2 diabetic patients, with biopsy-proved diabetic glomerular sclerosis (DGS), have increased plasma and urinary MDA. Using MDA glomerular immunostaining, he demonstrated that augmented urinary MDA is the consequence of increased MDA in the glomerulus, indicating that local lipid peroxidation may be one of the major causes of glomerulosclerosis in patients with DGS. However, Hermanns et al. (28) suggested that the urinary MDA level could be a reliable index of kidney damage.

Formation and accumulation of advanced glycation end products (AGEs) as the result of carbonyl and oxidative stress may produce changes in protein charge, solubility, and conformation leading to molecular dysfunction and resulting in the pathologic accumulation of collagens, fibronectins, and laminins (29, 30). The result of these interactions is the leaking of glomerular basal lamina and albumin appearance into urine (31). Reactive carbonyl groups in urine have not been studied in DN until now. In this study, higher values of RCD have been found in DTM2-MAA and DTM1-MIA diabetic groups compared to control subjects, but only when they are expressed as mM creatinine (*Figure 2*). Markedly increased values of RCD were obtained for DTM2-MAA patients indicating a higher degree of renal injury. Significantly different results were obtained if the values are expressed as mg protein in urine. In our opinion, the expression of RCD values as mg protein is not quite appropriate because of the large amount of proteines in the urine of patients with macroalbuminuria. Only when the level of RCD in urine is expressed on creatinine, it could reflect the degree of renal damage, and this parameter could be used as a diagnostic tool for DN.

The measurement of the urinary levels of NAG, a lysosomal enzyme abundant in proximal tubular segments, has been a useful marker for monitoring tubular damage in patients on nephrotoxic therapy, with glomerulonephritis and DN (32). Elevated urinary NAG excretion has been reported in renal damage produced by various causes, including diabetic humans and rats (33, 34). In this study we found elevated NAG and other studied enzymes in the DTM2-MAA patient group. The relationship between the investigated urinary enzymes activities and severity of proteinuria might be explained with the results of Bazzi et al. (35) and Hultberg et al. (36). They demonstrated that increased excretion of urinary enzymes in proteinuric glomerular diseases can occur even in the absence of morphological evidence of tubular cells damage, probably reflecting increased lysosomal activity of these cells due to an increased uptake and catabolism of filtered proteins. The activity of all enzymes in the urine is correlated with increased macroalbuminuria and other parameters relevant to diabetes. Therefore, these enzymes are an indicator of the functional status of the renal tubules as well as tubular injury (37, 38). Thus, the significant increase observed in the levels of MDA, urinary brush border enzymes and albumin excretion supports the hypothesis that oxidative stress induces tubular cell injury in diabetic patients.

The recognition that oxidative stress represents a key factor in the development of DN provides a lot of possibilities for advanced therapeutic strategies. It is likely that the prevention of diabetic renal injury, along with conventional approaches, will have antioxidant therapies as an important part of future therapy in these patients.

In conclusion, the level of oxidative stress markers and activities of brush border ectoenzymes in the urine may be a useful non-invasive and easily repeatable test for assessing an initial malfunction or damage of the proximal tubular epithelial cells in the early stages of potentially progressive diseases. Finally, it has a predictive value for the functional outcome and response to therapy.

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