

# Erythrocyte glutathione transferase activity: a possible early biomarker for blood toxicity in uremic diabetic patients

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**Abstract** Erythrocyte glutathione transferase (e-GST) displays increased activity in patients with renal damage and positive correlation with homocysteine (Hcy) in patients under maintenance hemodialysis. Here, we determined e-GST, Hcy, and erythrocyte catalase (e-CAT) in 328 patients affected by type 2 diabetes mellitus (T2DM), 61 diabetic non-nephropathic patients and 267 affected by diabetes and by chronic kidney disease (CKD) under conservative therapy subdivided into four stages according to K-DOQI lines. e-GST activity was significantly higher in all T2DM patients compared to the control group ( $7.90 \pm 0.26$  vs.  $5.6 \pm 0.4$  U/g<sub>Hb</sub>), and we observed an enhanced activity in all subgroups of CKD diabetic patients. No significant correlation or increase has been found for e-CAT in all patients tested. Mean Hcy in

diabetic patients is higher than that in healthy subjects ( $33.42 \pm 1.23$  vs.  $13.6 \pm 0.8$   $\mu$ M), and Hcy increases in relation to the CKD stage. As expected, a significant correlation was found between e-GST and Hcy levels. These findings suggest that e-GST hyperactivity is not caused directly by diabetes but by its consequent renal damage. e-GST, as well as Hcy, may represent an early biomarker of renal failure.

**Keywords** Diabetes · Erythrocyte glutathione transferase · Erythrocyte catalase · Homocysteine · Chronic kidney disease

## Introduction

The erythrocyte glutathione transferase (e-GST) is a member of a superfamily of inducible enzymes involved in cell protection and detoxification [1]. A prominent function of these enzymes is the conjugation of glutathione (GSH) to toxic hydrophobic compounds provided by an electrophilic center [2]. This reaction facilitates their inactivation and renal elimination of a large number of toxins [1]. Overexpression of e-GST has been found only in uremic patients under maintenance hemodialysis (MHD) [3] and in subjects affected by hyperbilirubinemia [4]. Recently, a study confirmed the enhanced e-GST activity in MHD patients, and for the first time, they also investigated e-GST activity in chronic kidney disease (CKD) patients under conservative therapy, observing a significant increase related to the K-DOQI stage [5]. In addition, e-GST has been proposed as an innovative biomarker for dialysis adequacy complementary to  $Kt/V_{\text{urea}}$  [6].

Type 2 diabetes mellitus (T2DM) is associated with increased production of reactive oxygen species (ROS) and

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a reduction in antioxidant defences leading to oxidative stress. ROS production induced by chronic hyperglycemia is implicated as a potential molecular mechanism behind diabetic vascular complications [7]. An important enzyme involved in the cell detoxification of peroxide is catalase, an enzyme present in all aerobic organisms [8].

Until now, relationship between T2DM patients and e-GST and catalase activities has never been investigated. The first aim of this study is to determine e-GST and e-CAT activities in a large number of patients affected by T2DM. These patients have been also divided into two distinct groups: diabetics not affected and affected by nephropathy under conservative therapy. This last group has been further divided into four subgroups according to the stage of renal failure using K-DOQI guidelines [9].

Finally, in the light of recent findings showing a significant correlation between the increased level of e-GST activity and plasmatic homocysteine (Hcy) concentrations in maintenance hemodialysis patients (MHD) [5], we assessed Hcy levels in all groups of patients.

Homocysteine (Hcy) is the transmethylation product of the essential sulfur-containing amino acid methionine [10]. Increased level of this amino acid may cause vascular damage, endothelial dysfunction and cardiovascular disease [11], which is the most common cause of mortality in patients with T2DM [12]. The association between homocysteinemia and atherosclerotic vascular disease is especially strong in patients with T2DM, compared to non-diabetic subjects. Previous studies demonstrated that plasma total homocysteine (tHcy) concentration is increased in CKD patients both under conservative therapy and in hemodialysis [13].

Here, we determined for the first time Hcy levels in patients affected by T2DM and chronic kidney disease under conservative therapy according to stage of CKD. In addition, we evaluated the possible correlation between plasmatic Hcy levels and e-GST and e-CAT activities.

## Materials and methods

### Patients and study design

The study protocol complied with the declaration of Helsinki (1964) and was appointed by the Ethical Committee of the University of Rome “Tor Vergata”. A written fully informed consent was provided by all patients and healthy subjects before enrollment into the studies.

Blood samples were obtained from 328 T2DM patients: 61 (46 men and 15 women) were not affected by nephropathy, and 267 were affected by CKD under conservative therapy. This last group has been subdivided in 4 subgroups, according to the stage of renal disease (using

K-DOQI guidelines) [9]: stage I (30 men and 30 women), stage II (31 men and 39 women), stage III (50 men and 20 women) and stage IV (32 men and 35 women). The GFR was evaluated using CKD-EPI formula [14].

Blood samples were obtained also from 82 healthy subjects (control group: 36 men and 46 women), with normal renal function ( $\text{GFR} \geq 90$  mL/min and absence of microalbuminuria and/or microhematuria) and not affected by diabetes mellitus, recruited from the Department of Occupational Medicine of “Tor Vergata” University, Rome.

Inclusion criteria for diabetic patients are the following: subjects older than 18 years, T2DM subjects with normal renal function and T2DM subjects with impaired renal function.

Exclusion criteria used for T2DM patients with normal renal function, for nephropathic patients and for healthy control subjects were the following: presence of solid tumors, hematological disease (whether oncological or not), infectious disease, autoimmune disease in active phase, liver disease (clinical history of B or C hepatitis, AST and/or AST serum levels two times higher than the normal range, hyperbilirubinemia), blood transfusion in the last 3 months and type I diabetes mellitus.

### Analytical procedures

All reagents in this study were from Sigma-Aldrich (St. Louis, USA) and used without further purification.

Blood samples were collected from the anticubital vein both in healthy subjects and in T2DM subjects with normal renal function or affected by CKD under conservative therapy.

For e-GST activity determinations, blood samples were collected from the anticubital vein and stored into K-3-EDTA tubes at 4 °C for no more than 2 days. One volume (40  $\mu\text{L}$ ) of whole blood was diluted in 25 volumes (1.0 mL) of bi-distilled water and after 2 min analyzed for GST activity determination. GST activity was assessed spectrophotometrically at 340 nm (37 °C) using 100  $\mu\text{L}$  of hemolyzed sample in 1 mL final volume containing 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1 M potassium phosphate buffer, pH 6.5. The molar extinction coefficient for CDNB conjugation product at 340 nm is  $9,600 \text{ M}^{-1} \text{ cm}^{-1}$ . Time run was 1 min. The spectrophotometric assay method for GST with slight modifications is similar to the one described in literature [15].

GST activity was expressed as enzyme units (U) per gram of hemoglobin (Hb). One unit represents the amount of enzyme that catalyzes the conjugation of 1  $\mu\text{mol}$  of GSH to CDNB in 1 min at 37 °C. Recombinant human GSTP1-1, used as internal standard, was expressed in *Escherichia coli* and purified as described in literature [16].

Even for catalase determinations blood samples were collected as above described. In this case, in order to

determine enzyme activity, we used 5  $\mu\text{L}$  of hemolyzed blood sample. Enzyme activity was measured spectrophotometrically at 240 nm (25 °C), evaluating the disappearance of hydrogen peroxide. Extinction coefficient for hydrogen peroxide at 240 nm is  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ . The assay test contained 5  $\mu\text{L}$  of hemolyzed sample, 10 mM  $\text{H}_2\text{O}_2$  in 0.05 M potassium phosphate buffer, pH 7.0 with EDTA  $10^{-4}$  M.

All routine laboratory parameters were determined using Dimension Vista 1500 (Siemens, Healthcare Diagnostics, Milano, Italy).

Hemoglobin was measured using an automatic hematologic analyzer XE2100 (Sysmex, Dasit, Milano, Italy).

For Hcy (3.70–14.00  $\mu\text{mol/L}$ ) assessment, blood samples were collected in K-3-EDTA vacutainer tubes, put on ice and immediately centrifuged and stored at  $-20$  °C until analysis. Plasma tHcy was assayed by a fully automated HPLC method using reverse-phase separation and fluorescence detection [17].

### Statistical analysis

After the analytical procedure, all patients' data have been analyzed. Data are expressed as mean  $\pm$  standard deviation of the mean (SEM). Unpaired *t* test was employed to compare the data between various groups; nonparameter variables were analyzed by Mann–Whitney test. A value of  $p < 0.05$  was considered statistically significant. Data were processed using statistical software MedCalc™ (Mariakerke, Belgium). Passing-Bablock regression analysis was performed according to the original protocol [18].

## Results

Clinical and epidemiological features of 82 healthy subjects (control group of 36 men and 46 women) and of 328

diabetic patients, 267 divided in four subgroups basis on stages of renal failure according to K-DOQI guidelines [9] (stage I, 30 men and 30 women; stage II, 31 men and 39 women; stage III, 50 men and 20 women; stage IV, 32 men and 35 women), and 61 not affected by nephropathy (46 men and 15 women) are summarized in Table 1.

Erythrocyte glutathione transferase activity evaluated in all T2DM patients ( $7.90 \pm 0.26 \text{ U/g}_{\text{Hb}}$ ) is significantly higher compared to healthy control subjects ( $5.6 \pm 0.4 \text{ U/g}_{\text{Hb}}$ ,  $p < 0.001$ ). The e-GST activity is enhanced in all subgroups of chronic kidney disease diabetic patients compared to control group; this rise appears to be related to the stage of CKD, reaching 90 % of increase in CKD IV stage (Fig. 1; Tables 2 and 3). Interestingly, the e-GST activity is significantly increased in T2DM patients not affected by renal failure (stage 0) versus healthy subjects ( $6.76 \pm 0.5$  vs.  $5.6 \pm 0.4$ ,  $p < 0.0476$ ) (Tables 2 and 3). This unexpected evidence will be discussed below and suggests that e-GST could be a very early marker of renal dysfunction. As the diabetic cohort differs from healthy subjects about BMI, age and other comorbidities we also verified if these factors may alter the e-GST expression. However, no correlation exists between e-GST and BMI parameter for healthy subjects ( $R^2 = 0.2$ ,  $p = 0.25$ ), total diabetic patients ( $R^2 = 0.08$ ,  $p = 0.73$ ) and also diabetic patients subdivided into CKD stages (e-GST vs. BMI for CKD subgroups:  $R^2 < 0.1$ ,  $p \gg 0.05$ ).

Erythrocyte glutathione transferase expression is an age-independent parameter [19]. As expected, we found no correlation between e-GST activity and age in healthy subjects ( $R^2 = 0.01$ ,  $p = 0.84$ ) and total diabetic patients ( $R^2 = 0.0003$ ,  $p = 0.81$ ) also subdivided into CKD stages ( $R^2 < 0.3$ ,  $p > 0.05$ ).

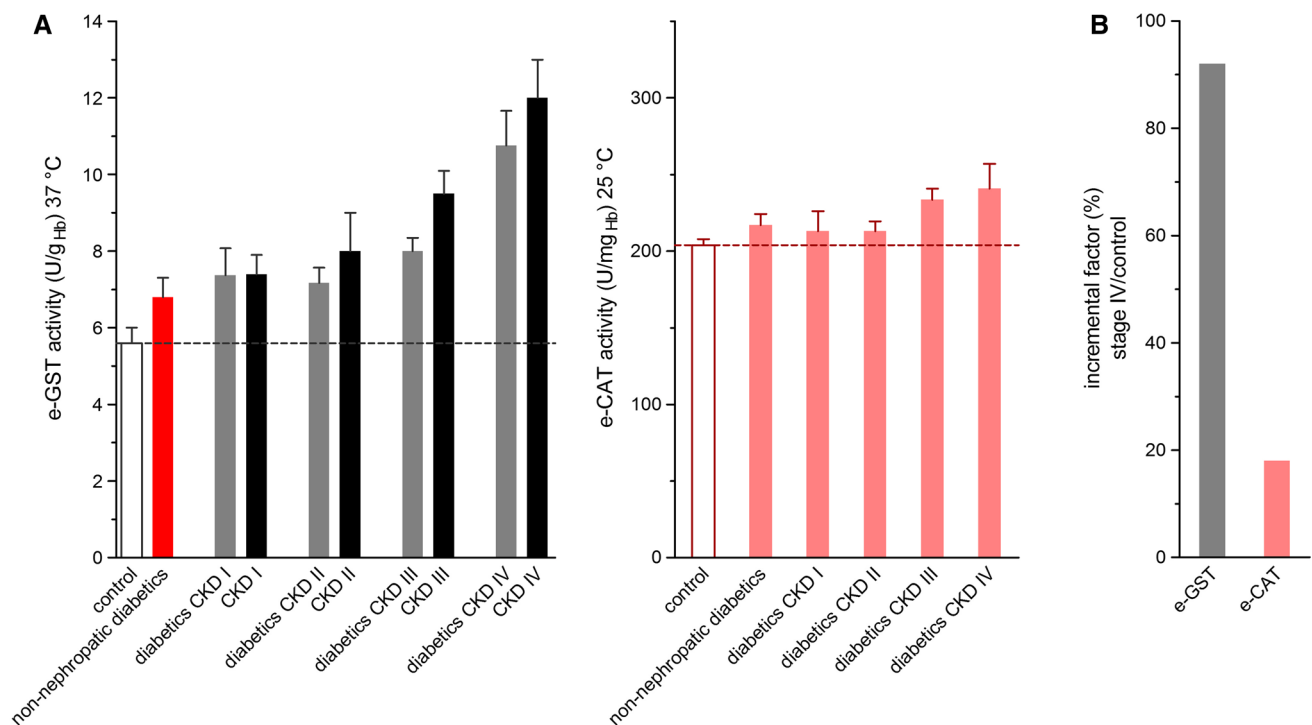
Finally, in our previous studies, no correlation has been also observed between e-GST activity and conventional markers of either acute (i.e., alpha-1 acid glycoprotein) or

**Table 1** Clinical and epidemiological features of study population

	Control group	Diabetic patients				
		0	1	2	3	4
Number	82	61	60	70	70	67
M/F	36/46	46/15	30/30	31/39	50/20	32/35
Age (years)	$44 \pm 2^*$	$63 \pm 2^*$	$70 \pm 1^*$	$72.5 \pm 0.9^*$	$74 \pm 1^*$	$74 \pm 1^*$
BMI	$19.0 \pm 0.4^*$	$32.4 \pm 0.8^*$	$30.3 \pm 0.5^*$	$30.1 \pm 0.6^*$	$29.3 \pm 0.5^*$	$29.6 \pm 0.5^*$
GFR (CKD-EPI) (mL/min)	$117 \pm 2^*$	$107 \pm 3^*$	$100 \pm 1^*$	$74.9 \pm 0.9^*$	$43 \pm 1^*$	$21.9 \pm 0.6^*$
Hypertension (%)	0	91.80	98.33	97.14	97.14	100
Dyslipidemia (%)	0	50.82	39.34	72.86	71.43	94.03
Diabetes (%)	0	100	100	100	100	100

BMI body mass index, GFR glomerular filtration rate

\* Data are expressed as mean  $\pm$  standard error of the mean (SEM)



**Fig. 1 a** e-GST (left) and e-CAT activity (right) in patients subdivided on the basis of CKD stages. Dotted line represents the mean value of the control group (healthy subjects). Black columns (left) are the e-GST activity of nephropathic non-diabetic patients

previously reported in literature [5]. All values are reported as mean  $\pm$  SEM. **b** Incremental factor (%) of e-GST and e-CAT activities found in diabetic patients (CKD stage IV) compared to healthy subjects (taken as 0 %)

chronic inflammation (i.e., hs-CRP), or chronic inflammation/kidney disease (i.e., beta-2 microglobulin) and malnutrition-inflammation (i.e., prognostic inflammatory and nutritional index) [5].

No significant increase in e-CAT has been found for T2DM patients and no correlation with the stage of CKD. Moreover the e-CAT activity shows a high inter-individual variability (Fig. 1; Table 2).

Mean Hcy values in diabetic patients resulted higher than in healthy subjects ( $33.42 \pm 1.23$  vs.  $13.6 \pm 0.8$   $\mu\text{mol/L}$ ,  $p < 0.001$ ). Homocysteine seems to follow an increasing trend in relation to the stage of CKD (stages I–IV according K-DOQI guidelines) [9], and this increase would appear to contribute not only diabetes but also the stage of CKD (Tables 2 and 3). Furthermore, a significant correlation has been founded between e-GST activity and Hcy value ( $R^2 = 0.86$ ,  $p = 0.008$ ) (Fig. 2).

## Discussion

Erythrocyte glutathione transferase and e-CAT activities in T2DM patients affected by CKD under conservative therapy and in diabetic patients not affected by nephropathy have been evaluated through simple spectrophotometric assays. In particular, the e-GST assay procedure, validated

**Table 2** Erythrocyte glutathione transferase and e-CAT activity and Hcy value in healthy control subjects and in diabetic patients, divided in subgroups according to CKD stage

	e-GST (U/gHb) <sup>a</sup>	Hcy ( $\mu\text{mol/L}$ ) <sup>a</sup>	e-CAT (U/gHb) <sup>a</sup>
Control group	$5.6 \pm 0.4$	$13.6 \pm 0.8$	$203,950 \pm 3,860$
Diabetic patients			
Non-nephropathic	$6.8 \pm 0.5$	$25 \pm 2$	$217,011 \pm 7,360$
CKD stage I	$7.1 \pm 0.8$	$26 \pm 4$	$212,460 \pm 13,090$
CKD stage II	$7.2 \pm 0.4$	$27 \pm 2$	$213,110 \pm 6,450$
CKD stage III	$8.0 \pm 0.3$	$39 \pm 2$	$233,480 \pm 7,310$
CKD stage IV	$10.8 \pm 0.9$	$44 \pm 4$	$240,740 \pm 16,290$

<sup>a</sup> All data are expressed as mean  $\pm$  standard error of the mean (SEM)

previously by classical recovery experiments, i.e., linearity, interday and intraday dosage has been used through this study [5].

Our results show, for the first time, a significant increase in e-GST activity in diabetic patients, which reaches its highest values in the CKD stages III and IV, evaluated using K-DOQI guidelines. A similar result has been obtained in a previous study on nephropathic patients not affected by diabetes mellitus [5] (Fig. 1). Obviously, the observed increase in e-GST could be caused by diabetes itself or by the renal failure due to diabetes or by both these

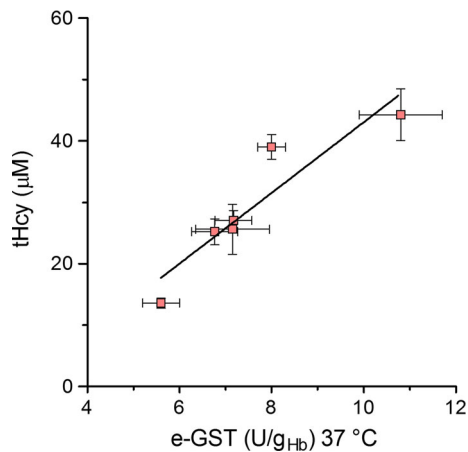
**Table 3** Statistical correlation of e-GST activity and Hcy values between control group and all stages of diabetic patients

	Diabetic patients (e-GST)				
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Control group (e-GST)	$p = 0.047^{**}$	$p < 0.050^{**}$	$p < 0.050^{**}$	$p < 0.001^{**}$	$p < 0.001^{**}$
Diabetic patients (e-GST)					
Stage 0		$p = 0.460^*$	$p = 0.935^{**}$	$p = 0.084^{**}$	$p = 0.003^{**}$
Stage 1			$p = 0.896^{**}$	$p = 0.344^{**}$	$p = 0.024^{**}$
Stage 2				$p = 0.025^{**}$	$p = 0.0002^{**}$
Stage 3					$p = 0.010^{**}$
	Diabetic patients (Hcy)				
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Control group (Hcy)	$p < 0.0001^{**}$	$p = 0.002^{**}$	$p < 0.0001^{**}$	$p < 0.0001^{**}$	$p < 0.0001^{**}$
Diabetic patients (Hcy)					
Stage 0		$p = 0.938^{**}$	$p = 0.861^{**}$	$p < 0.0001^{**}$	$p < 0.0001^{**}$
Stage 1			$p = 0.657^{**}$	$p = 0.016^*$	$p = 0.011^{**}$
Stage 2				$p < 0.001^{**}$	$p < 0.001^{**}$
Stage 3					$p = 0.392^{**}$

$p$  value  $< 0.05$  is considered statistically significant

\* Independent samples  $t$  test

\*\* Mann–Whitney test



**Fig. 2** Correlation between e-GST ( $U/g_{Hb}$ ) and total Hcy ( $\mu M$ ) in healthy subjects, nephropathic and non-nephropathic diabetic patients ( $R^2 = 0.86$ ,  $p = 0.008$ ) as reported in Table 2

factors. Our data seems to discriminate among these possibilities. In fact, we observed that e-GST activity in T2DM patients not affected by nephropathy (stage 0) is significantly higher than that found in the healthy subjects. If this overactivity is due to diabetes alone, a similar surplus of activity is expected in all CKD stages and must implement the hyperactivity due to the nephropathy. On the contrary, the values found in diabetic patients at the CKD stages II, III and IV are comparable and even lower to that found in non-diabetic nephropathic patients (Fig. 1). Thus, the

possibility that diabetes alone could induce the e-GST hyperexpression is unlikely. A stimulating hypothesis is that the observed increase in e-GST found in non-nephropathic T2DM patients could be due to a masked defective function of kidney. In this case, e-GST may represent an early biomarker of renal damage for diabetic patients, even before the onset of microalbuminuria. Interestingly, we also found a mean plasma Hcy value significantly higher in diabetic patients, compared to healthy subjects. Hyperhomocysteinemia is considered a cardiovascular risk factor, which is often associated with diabetes mellitus and chronic kidney disease [20]. Furthermore, we observed a significant correlation between e-GST activity and Hcy values (Fig. 2). These findings are in agreement with previous observations obtained in studies on CKD patients in MHD, not affected by diabetes mellitus [5]. Curiously, it has been observed a homocysteine increase in diabetic patients without evident nephropathy, and this behavior parallels what found for e-GST (Tables 2 and 3). Therefore, both Hcy and e-GST could represent similar early biomarkers which reveal renal failure not visible by traditional analyses.

In this study, we also examined another enzyme involved in cell detoxification: erythrocyte catalase. However, for this enzyme, we did not find any possible correlation with the gravity of renal diseases in T2DM patients and very similar values in all CKD stages (Fig. 1; Table 2). Therefore, evaluation of e-CAT activity in diabetic patients

has not proved to be of great utility for diagnostic purposes, unlike e-GST activity, which on the contrary can be considered a new biomarker of oxidative stress and of its related comorbidities, due to the progressive increase observed starting from stage 0 to stage IV of CKD according to K-DOQI guidelines [9].

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**Conflict of interest** The authors declare no conflict of interest.

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