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Urinary Markers for Early Detection of Diabetic Nephropathy in Type 1 Diabetes Mellitus

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ABSTRACT

Background: diabetic nephropathy (DN) is a serious complication of diabetic mellitus associated with increased risk of morbidity and mortality. Diagnostic markers to detect DN at early stage are important as early intervention can slow loss of kidney functions and improve patient outcomes. N-acetyl-β-d-glucosaminidase (NAG) is a lysosomal enzyme, present in high concentrations in renal proximal tubular cells, Gamma-glutamyltransferase (GGT) is an enzyme which located along the proximal tubular brush border, Malondialdehyde (MDA) is a highly toxic product, formed in part by lipid oxidation derived free radicals, Reactive carbonyl derivatives (RCD_S) is an oxidative stress marker in urine, as a measure of the oxidative modification of proteins and bete-2-microglobulin is filtered by the glomerulus, absorbed and catabolized by the proximal tubules. The aim of this study is to investigate the urinary outcome of these markers as early detectors of diabetic nephropathy in type 1 diabetic children.

Subjects and methods: This case-control study included 67 children with type 1 diabetes mellitus (33 male; 34 female), age (**11.03±1.05** years) and thirty one age (**10.58±1.11 years**) and sex (13 male; 18 female) matched healthy children (13 male; 18 female). Type 1 diabetic children were further subdivided into microalbuminuric and normoalbuminuric subgroups according to microalbuminuria concentration (30 mg/ g creatinine). Age, sex, diabetic duration and the current daily insulin dose, and family history of diabetes, weight, height, body mass index, systolic and diastolic blood pressure were recorded. Fasting plasma glucose, glycated hemoglobin, blood urea nitrogen, plasma creatinine, urinary creatinine, micoalbumin, N-acetyl-B-D glucosaminidase (NAG), Gama glutamyl transferase (GGT), Beta-2-microglobulin, Malondialdehyde (MDA) and Reactive carbonyl groups ($RCDS_S$) were measured in all subjects.

Results: a significant increase in tubular injury markers of diabetes (NAG, GGT, beta-2-microglobulin) and oxidative stress parameters (MDA, $RCDS_S$) as compared to control subjects was found. Microalbuminuric subjects showed a significant elevatation in the urinary markers including NAG, GGT, beta-2-microglobulin, MDA, $RCDS_S$ as compared to normoalbuminuric subjects. The studied urinary tubular enzymes (NAG, GGT), oxidative stress markers (MDA, $RCDS_S$) and Beta-2- microglobulin showed positive correlations with one another. **Conclusion**: The results of this study introduced the possibility of depending on tubular enzymes (NAG, GGT), oxidative stress markers (MDA, $RCDS_S$) and β_2 microglobulin as early, reliable, and sensitive predictors for diabetic nephropathy. The NAG activity index proved to be the most sensitive biomarker, then beta-2- microglobulin for early discovering the tubule cells damage.

Keywords: Diabetic nephropathy- Micralbuminurea-Type 1 diabetic children- Glucose- N-acetyl-B-D glucosaminidase (NAG), Gama glutamyl transferase (GGT), Beta-2-microglobulin, Malondialdehyde (MDA) - Reactive carbonyl groups ($RCDS_S$)

INTRODUCTION

Diabetic nephropathy is a severe complication occurring in diabetic patients and it is associated with an increased risk of mortality, cardiovascular disease and progression to end stage renal disease (ESRD). [(1)] It has demonstrated that the onset and course of DN can be ameliorated significantly by several interventions, but these interventions have their greatest impact if instituted at a very early stage in the course of the development of DN.[(1).]

Diagnostic marker to detect DN at early stage is important as early intervention can slow the loss of

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kidney function and reduce adverse outcomes. [2]. Microalbuminuria is considered the first sign and the best predictor of progression to renal failure and cardiovascular events. However, albuminuria has several limitations. Therefore, there is an intensive effort invested in the search for early specific urine biomarkers of glomerular and tubular injury in diabetic nephropathy.

N-acetyl-beta-D-glucosaminidase (NAG) is a lysosomal enzyme present in high concentrations in renal proximal tubular cells. [3] 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. [4].

Gama glutamyl transferase (GGT) is located along the proximal tubular brush border. [5] Emerging evidence suggests GGT as a predictor of incident diabetes and Hypertension. Few studies have also shown that GGT predicts microalbuminuria and may also act as a predictor of microvascular complications in diabetes patients. [5]

Beta2-microglobulin is a low molecular weight protein normally cleared by the kidneys at a rate comparable to glomerular filtration rate (GFR), then reabsorbed and catabolized in the tubules and serum levels are inversely related to GFR. [6] Urinary beta 2 microglobulin appears to be sensitive in detecting an abnormality of the renal proximal tubule which may be an early feature of diabetic renal involvement not characterized by microalbuminuria. [7]

Oxidative stress is considered to be a unifying link between diabetes mellitus and its complications, including nephropathy. [8]

Degradation of lipid peroxides generates different compounds, such as malondialdehyde (MDA) which may be transported or simply leak from the organ or tissue into the bloodstream and become excreted in urine. Just one oxidative stress markers in urine is reactive carbonyl derivatives (RCD_S), as a measure of the oxidative modification of proteins. [8]

The aim of this study was to investigate the association between the urinary enzyme activities of NAG and GGT as well as some oxidative stress markers (MDA, RCDs) in type 1 diabetic children and the severity of microalbuminuria. Furthermore, the study aimed to answer the question whether urinary output of these parameters can be used as renal dysfunction screening markers in type 1 diabetic children.

SUBJECTS AND METHODS

This study included 67 children with type 1 diabetes mellitus (33 male; 34 female), age (5 years-12 years) who were recruited from outpatient diabetic clinic of the National Institute for Diabetes and Endocrinology, Cairo, Egypt. All patients met the criteria of American Diabetes Association for type 1 diabetes. [9] Written informed consent was obtained from the guardians, and this study was approved by the Ethics Committee of the National organization for teaching hospitals and institutes. Type 1 diabetic patients were matched with another group of 31 (13 male; 18 female) control healthy subjects in terms of age, sex and socioeconomic status. Patients were further subdivided into two groups according to microalbuminuria concentration: 31 microalbuminuric patients (30 mg/ g creatinine) and 36 normoalbuminuric patients (< 30mg/ g creatinine). A full medical history including age, sex, diabetic duration and the current daily insulin dose were recorded on all normal and diabetic subjects. Exclusion criteria included those who were receiving any medication other than insulin and those who were complaining from any acute or chronic illness other than diabetes mellitus.

Preparation of samples and biochemical analyses

1-Blood samples

Venous blood samples were taken from all subjected after 10-12 hour fasting into fluoride-oxalate tubes and heparinized tubes. Fluoride-oxalate tubes were immediately centrifuged at 3000 r.p.m for 15 minutes and the separated plasma was used for determination of fasting plasma glucose. [10] **An aliquot of fresh heparinized blood was used for the determination of % of glycated hemoglobin according to Abraham *et al.*** [11] and plasma creatinine concentration (mg/dL) according to Fabiny and Eringhausen. [11] The rest of plasma aliquots was frozen at – 40ºC until determination of blood urea nitrogen (mg/dL). [12]

2-Urine samples

First morning void urine samples (midstream) were taken from all subjects one portion was immediately used for determination of creatinine concentration (mg/dl), microalbuminuria concentration (mg/g creatinine) Cambiaso *et al*.[13] , gama glutamyl transferase activity (GGT) (U/ g creatinine). [14] Another portion was kept frozen at -80ºC until further determination of N-acetyl-B-D-glucosaminidase (NAG) (µmol/ gm creatinine) as described by Horak *et al*. [15]. Malondialdehyde (MDA) (µg/g creatinine) according to Goulart *et al*. [16] Reactive carbonyl groups ($RCDS_s$) (µmol/ g creatinine) Leving *et al*. [17] and beta-2-Microglobulin (β_2-M) (µg/g creatinine) using an Immunometric Enzyme Immunoassay kit provided by Orgentec Diagnostika GmbH (USA). [18].

Statistical Analyses

Data were collected, checked, revised and entered the computer. Data were analyzed by SPSS statistical package version 17. Excel computer program was used to tabulate the results, and represent it graphically. Independent t-test was used to declare the significant difference between each two groups at $p<0.05$. Pearson correlation coefficient at $p<0.05$ was used to declare the significant correlation between the variables within each group. [19]

Urinary albumin:creatinine ratio, NAG:creatinine ratio and GGT:creatinine ratio, MDA :creatinine ratio, RCDS:creatinine ratio and urinary beta-2-microglobulin:creatinine ratio were shown to be logarithmically normally distributed by normal order plots.5 Results were therefore transformed logarithmically before analysis.

RESULTS

Clinical characteristics of healthy control, and type 1 diabetic children were shown in (table 1), there was no significant differences between diabetic and healthy subjects concerning age; weight, height; body mass index and blood pressure (systolic and diastolic). On comparing between diabetic with respect to normalbuminuria and microalbuminuria, it was found that there were no significant differences in age, diabetic duration, daily insulin dose, weight, height, and body mass index, systolic and diastolic blood pressure.

Results illustrated in table (2) showed that type 1 diabetic group showed a highly significant increase in the levels of fasting plasma glucose (89·2%) while, urinary creatinine concentration of diabetic children was significantly lower (42·3%) than that of the healthy control children. However, there were no significant differences for plasma creatinine and blood urea nitrogen between the two studied groups.

On comparing glycemic control parameters of normoalbuminuric and microalbuminuric type 1 diabetic patients, no significant difference was found between the two groups. Meanwhile, kidney function parameters of the two groups showed that urinary creatinine was decreased in microalbuminuric group than normoalbuminuric group; (0.36±0.083g/L vs 0.95±0.19) and the level of urea nitrogen was elevated in microalbuminuric comparing with normoalbuminuric group; (21.56±4.11 vs 16.16±3.86g/L)While, plasma creatinine showed no significant difference between the two groups (table 2).

Data shown in table (3) revealed that albumin execration of type 1 diabetic group was increased by 35·5% as compared to healthy control group. Moreover, there were significant increases ($p<0.05$) in tubular injury markers of diabetic group with percentages of 15·2%; 137·8% and 42·3% for GGT; NAG and beta-2-microgloulin respectively as compared to the healthy control group. Meanwhile, oxidative stress parameters were significantly higher in patient compared to control ($P<.05$). Among type 1 diabetic group; albumin/creatinine output was significantly elevated by 72·53% in microalbuminuria diabetic patient as compared to the normoalbuminuria diabetic ones. In addition, the other assayed markers were also significantly elevated in the former group as compared to the latter ones (table3).

DISCUSSION

Diagnosis of diabetic nephropathy in the early stages is very important since there are no clinical signs or symptoms. Therefore, detection of reliable predictors of diabetic nephropathy is desirable because this complication may be reversed or delayed by strict glycemic control and treatment of hypertension in the early stages of nephropathy. [1]

The results of the present study revealed a significant increase in the urinary NAG activity in-patients with type 1 diabetes mellitus compared with control subjects ($p< 0.05$). This finding is in accordance with other studies [20, 21;22] Abdel Shakour *et al*. [23] stated that changes in urinary NAG activity can reflect the activity of diabetes as well as the residual functional capacity of the kidney. Kuzniar *et al*. [24] denoted that in proteinuric glomerular diseases the increased NAG excretion may reflect increased lysosomal activity of tubular cells due to the increased uptake of filtered proteins.

There was a positive correlation between NAG/creatinine ratio and microalbuminuria in type 1 diabetic subject (Figure, 1). This this coincides with finding of Kern *et al*. [25] who reported that early in type 1 diabetes, repeated measurements of albumin excretion rate (AER) and urinary NAG may identify individuals susceptible to future diabetic nephropathy, and that combining these two markers may yield a better predictive model than either one alone.

The activity of NAG was increased by 2 .0 times (0.912 ± 0.048U/g creatinine vs 1.534 ± 0.088U/g. creatinine) and 3.5 times (0.45 ± 0.03U/g. creatinine) of the control group in normoalbuminuric and microalbuminuric diabetic respectively. Navarro *et al*. [26] stated that NAG changes occur prior to microalbuminuria, probably because the tubular cells can reabsorb the increased albumin load that results from glomerular affection but the increased NAG will be lost from the damaged cells. Also, Nauta *et al*. [27] showed that urine NAG increases a surprising 9-fold in normoalbuminuric patients with diabetes compared to controls.

Gamma glutamyl transferase (GGT) in urine originates from the surface of brushy border of epithelial cells membrane in the proximal tubules lumen. It is a specific and sensitive indicator of these cells damage. [28] An increased urinary GGT was observed in the patients with diabetes without signs of renal function disorder, or even microalbuminuria. [29] This agrees with our finding which indicated significant increase in urinary GGT excretion of type 1 diabetic either normoalbuminuria or microalbuminuria group as comparing to healthy control. This enzyme excretion was connected with the duration of glycemia, degree of renal function damage. [29] Increasing excretion of urinary GGT may contribute to damaging in tubular cells. Jung *et al*. [30] reported that GGT is located in the brush-border membrane of the nephron. The location and function of the brush-border membrane make it a good target for the primary involvement in the pathogenesis of diabetic renal complications. Structural alterations of the diabetic brush-border membrane such as an increase in protein oxidation and lipid peroxidation with a reduction in fluidity result in functional changes in membrane associated activity of gamma glutamyl transferase. When the tubular cells are damaged, they release these enzymes into ultra-filtrate and thus the enzyme activities in urine increase. [31] Gatua *et al*. [32] suggested that site-specific urinary biochemical markers provide valuable information about early renal proximal tubular insult that ultimately may precede glomerular permeability in subjects with diabetes mellitus.

Beta 2- microglobulin is a low molecular weight protein that is released at constant
rate and filtered by the glomerulus, absorbed and catabolized by proximal tubules. Therefore, it is theoretically considered a suitable biomarker of renal dysfunction. In fact, tubular involvement may precede glomerular involvement because several of these tubular proteins and enzymes are detectable even before the appearance of microalbuminuria and rise in serum creatinine. [33]

Urinary beta-2-microglobulin is a sensitive marker of increased glomerular filtration and proximal renal tubular function. Chiaramonte *et al*. [34] stated that elevated urinary microprotein (beta-2-microglobulin) might be a useful marker of renal injury in children. Alteration in beta-2-microglobulin concentration has been observed in patients with different diseases, including diabetic nephropathy. [35] The increased levels of beta-2-microglobulin in diabetic children observed in the present study were co-inside with the results of Zeng *et al*. [36] who found that urinary β2-microglobulin was significantly increased in patients with tubular cell injury indicating that urinary β2-microglobulin is a sensitive (sensitivity,86.6%) assay for detecting tubular injury. They also demonstrated that measurement of urinary β2-microglobulin was a reliable assay to detect proximal tubule injury. Moreover, other

investigators [37,38] stated that beta-2-microglobulin and retinol binding protein (RBP) were higher in diabetic patients than in controls. Increased excretion of these proteins implies injury to the brush border membrane with loss of microvillous structure. Loss of a significant fraction of the microvillus surface area also leads to reduced reabsorption and increased excretion of filtered proteins. [39]

In this study, there was a positive correlation between NAG/creatinine ratio and beta-2-microglobulin / creatinine (Figure, 2). This finding agrees with Fathy *et al*. [38] who reported a significant positive correlation between urinary beta-2- microglobulin and urinary NAG excretion.

In the present study significantly elevated levels of urinary MDA and $RCDS_S$ were observed in diabetic children especially those having microalbuminuria. Similar findings have been previously reported by others [40;41]. Several studies have shown that increased lipid peroxides and/ or oxidative stress are present in diabetic subjects. [42,43]. Oxidative stress can be increased before clinical signs of diabetic complications [44]. In diabetes, oxidative stress seems to be caused by both increased production of reactive oxygen species (ROS), sharp reduction in antioxidant defenses and altered cellular redox status. [45] Hyperglycemia may lead to an increased generation of free radicals via multiple mechanisms. Oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities. [46]

Malondialdehyde (MDA) is a highly toxic product formed in part by lipid oxidation derived free radicals. Many studies have shown that its concentration is considerably increased in diabetes mellitus, correlating with poor glycaemic control [47,48] . Lipid peroxide-mediated tissue damage has been observed in type I and type II diabetes [46].

Carbonyl stress explains increased modification of proteins in diabetes, uremia and other diseases by a generalized increase in the concentration of reactive carbonyl precursors of AGEs, glycoxidation and lipoxidation products [49]**.** So, these carbonyls may damage not only proteins but also phospholipids and nucleotide base DNA.

Protein peroxidation can occur by various mechanisms: (i) an increase in the production of reactive oxygen species (ROS) (ii) decrease in the rate of scavenging of ROS, (iii) an increased susceptibility of the protein to oxidation and (iv) a decrease in the rate of removal of oxidized species [50]. Previous studies have shown that levels of Reduced glutathione (GSH) and superoxide dismutase (SOD) were decreased in diabetic patiants. [51,52] Also Domínguez *et al*. [53] reported that erythrocyte glutathione peroxidase was significantly lower in diabetic children compared with control subjects. This could be the possible reason for the elevated protein carbonyl content in diabetic patients [54]

A significantly higher level of MDA in the urine of type 1diabetic patients with microalbuminuria has been demonstrated by Cvetković *et al*. [41]) and this may confirm the current results. Some compounds generated as the product of lipid peroxides degradation (alkanes, alkenals, and hydroxyalkanes) could be transported into the bloodstream, becoming excreted in urine. [55] Chang *et al*. [56] has shown that diabetic patients, with biopsy-proved diabetic glomerular sclerosis (DGS), have increased plasma and urinary MDA. Moreover, Hermanns *et al*. [57] suggested that the urinary MDA level could be a reliable index of kidney damage.

In the current study, there was a significant difference in the measured urinary parameters including NAG, GGT, MDA, RCDSS and beta-2-microglobulin between the normoalbuminuric and microalbuminuric diabetic groups. These results may be confirmed by the findings of Gatua *et al*. [32], who reported that urinary enzyme levels were significantly higher in the microalbuminuric compared to normoalbuminuric patients, while urinary microprotein, serum creatinine and urea levels remained within the normal ranges. Other investigators suggested that proximal tubular biomarkers were significantly increased among the subjects exposed to risks of renal injury and were better markers of renal dysfunction. [58; 59]

The studied urinary tubular enzymes (NAG, GGT), oxidative stress markers (MDA, RCD_S) and beta-2-microglobulin showed positive correlations with one another (Figure 3, 4) because of their high sensitivity and originate from the same part of the kidney. Hyperglycemia leads to enhanced reactive oxygen species production, and as a result tubular cell damage and hence development of abnormal urinary enzyme excretion. The findings are also supported by other studies. [58; 59]

Increased urinary enzymes excretion may suggest tubular dysfunction and their detection could be useful for assessing the pre-clinical stage of diabetes nephropathy. [(60)]

The results of this study indroduced the possibility of depending on tubular enzymes (NAG, GGT), oxidative stress markers (MDA, RCD_S) and β2 microglobulin as an early, reliable, and sensitive predictors for the children diabetic nephropathy. The NAG activity index was proved to be the most sensitive parameter, then beta-2-microglobulin for early discovering the tubule cells damage.

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Table (1): Clinical characteristics of the healthy control and type 1 diabetic children normoal buminuric and microalbuminuric

Parameters	Control (n= 31) Mean ±S.D	Patients (n= 67) Mean ±S.D	Normoalbuminuric (n= 36) Mean ±S.D	Microalbuminuric (n= 31) Mean ±S.D
Age (years)	**10.58±1.11**	**11.03±1.05**	11.31±**1.06**	10.56±**1.71**
Diabetic duration (years)	-----	2.49±2.15	2.32±2.14	3.01±2.38
Daily insulin dose (units)	-----	38.06±6.72	37.72±5.12	39.56±8.54
Weight (Kg)	38.06±8.68	37.63±7.25	38.45±8.91	36.69±8.82
Height (m)	1.32±0.21	1.37±0.16	1.38±0.16	1.35±0.16
Body mass index (Kg/m^2)	21.09±3.46	20.00±4.65	20.30±4.87	20.00±4.34
Systolic blood pressure (mmHg)	110.00±8.16	108.43±7.94	107.84±7.81	110.62±6.8
Diastolic blood pressure (mmHg)	73.0±1.4	74.023±4.94	74.09±4.97	74.38±5.12

S.D: Standard deviation
Data are expressed as mean ± SD

Table (2): Glycemic control indices and kidney function tests of the healthy control and type1 diabetic children (normoalbuminuric and microalbuminuric

Parameters	Control (n= 31) Mean ±S.D	Patients (n= 67) Mean ±S.D	Normoalbuminuric (n= 36) Mean ±S.D	Microalbuminuric (n= 31) Mean ±S.D
Fasting plasma glucose (mg/dl)	90.34±7.34	170.89±39.9*	166.01±40.96	164.22±38.64
Plasma creatinine (mg/dl)	0.49±0.16	0.48±0.19	0.50±0.17	0.43±0.05
Urinary creatinine (g/L)	1.37±0.24	0.79±0.21*	0.95±0.19	0.36±0.083**
Blood urea nitrogen (mg/dl)	17.7±2.35	17.49±3.94	16.16±3.86	21.56±4.11**

Data are expressed as means ±SD

* : There is significant difference between patients and control by using independent t. test at P<0.05.

**: There is significant difference between Normoalbuminuric and Microalbuminuric groups by using independent t. test at P<0.05.

Table (3): Urinary markers of tubular and glomerular injury of the healthy control and type 1 diabetic children (normoalbuminuric and microalbuminuric) (Data are log transformed)

Parameters	Control (n= 31) Mean ±S.E	Patients (n= 67) Mean ±S.E	Normoalbuminuric (n= 36) Mean ±S.E	Microalbuminuric (n= 31) Mean ±S.E
Microalbuminuria (mg/g creatinine)	0.93 ± 0.10	1.26 ±0.05*	1.052 ± 0.03	1.815 ± 0.067**
GGT (U/g creatinine)	1.32 ± 0.01	1.52 ±0.02*	1.465 ± 0.018	1.703 ± 0.068**
NAG (U/gm.creatinine)	0.45 ± 0.03	1.07 ±0.05*	0.912 ± 0.048	1.534 ± 0.088**
β-2- microglobulin (µg/g.creatinine)	1.56 ± 0.11	2.22 ± 0.08*	2.051 ± 0.079	2.718 ± 0.1499**
MDA (mol/g.creatinine)	− 0.13±0.08	0.15 ±0.05*	-0.014 ± 0.043	0.558 ± 0.0803**
RCG_s (µmol/g.creatinine)	1.63 ± 0.04	1.94 ±0.04*	1.809 ± 0.039	2.279 ± 0.0402**

Data are expressed as Mean ± SD

*: There is significant difference between patients and control by using independent t. test at P<0.05.

**: There is significant difference between Normoalbuminuric and Microalbuminuric groups by using independent t. test at P<0.05.

GGT: gama glutamyl transferase, NAG: N-acetyl-B-D-glucosaminidase. MDA:Malodialdehyde, RCGs: Reactive carbonyl groups

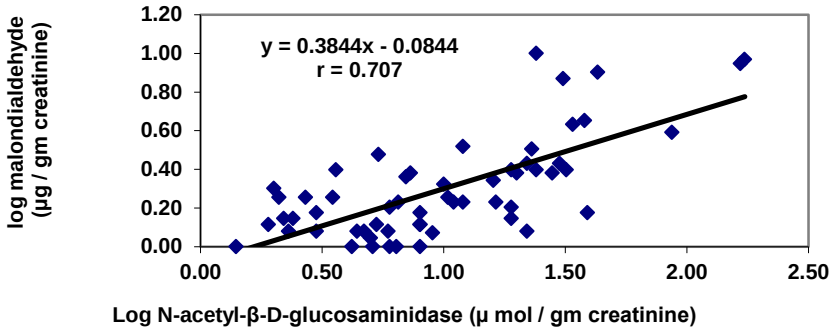

Figure (1): Correlation between log-transformed NAG and urinary log-transformed MDA in type 1 diabetic children

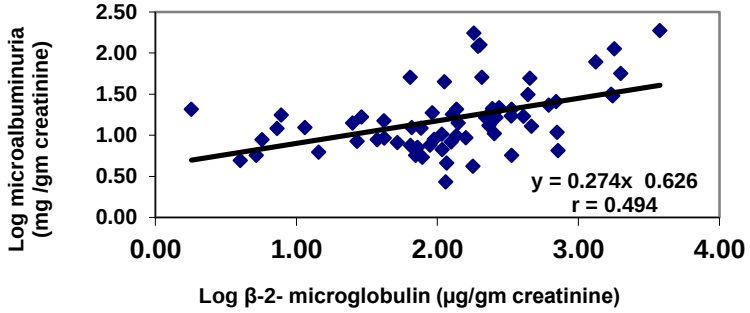

Figure (2) Correlation between log-transformed beta-2-microglobulin and urinary log-transformed microalbuminuria in type 1 diabetic children

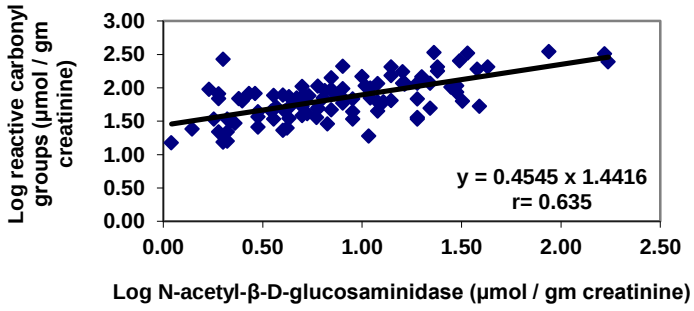

Figure (3): Correlation between log-transformed NAG and urinary log-transformed RCDs in type 1 diabetic children

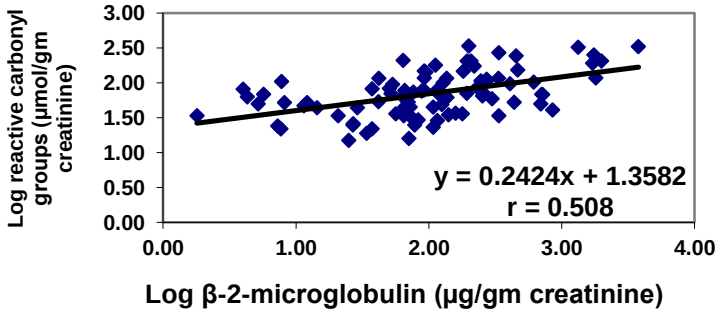

Figure (4) Correlations between log-transformed beta-2-microglobulin and urinary log-transformed RCDs in type 1 diabetic children