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URINARY N-ACETYL BETA GLUCOSAMINIDASE AND GAMMA GLUTAMYL TRANSFERASE AS EARLY MARKERS OF DIABETIC NEPHROPATHY.

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ABSTRACT

Albumin and enzymes- N-acetyl-beta-glucosaminidase (NAG) and gamma glutamyl transferase (GGT) were estimated in the morning random urine samples of 196 albustix negative diabetic patients to evaluate the clinical utility of these urinary enzymes as early markers of diabetic nephropathy. Albumin was estimated by immunoturbidimetric method and enzymes by kinetic essay within six hours of voiding of urine. The urinary albumin and urinary enzyme concentration was calculated in terms of ratio with respect to urinary creatinine. Correlation coefficient (r) between urinary albumin and urinary enzymes in normoalbuminuric, microalbuminuric and overall diabetic cases was 0.23, 0.32 and 0.40 respectively for NAG, and 0.08, 0.06 and 0.18 respectively for GGT. NAG excretion was found increased in 34%, 63.7% and 49.5% of normoalbuminuric, microalbuminuric and overall diabetic cases respectively while GGT in 6.4%, 24.5% and 15.8%. The correlation coefficient between urinary albumin and NAG in normoalbuminuric, microalbuminuric, and overall diabetic patients with increased NAG excretion was found only 0.31, 0.27 and 0.35 respectively. No correlation was found between duration of diabetes and enzyme excretion. The study suggests that urinary NAG or GGT or both together do not have any clinical significance as an early marker of diabetic nephropathy.

KEY WORDS

Microalbuminuria, Diabetic nephropathy, N-acetyl-beta-glucosaminidase, Gamma glutamyl transferase

INTRODUCTION

Diabetes mellitus is a global problem with approximately 150 million diabetic patients. This chronic condition poses a five times greater risk for developing nephropathy and has become the leading cause of end stage renal disease ESRD (1). Almost one third of the diabetic patients develop diabetic nephropathy in their life time (2).

Diabetic nephropathy is defined as persistent albuminuria detected by various dipsticks (Albustix positive or clinical albuminuria) and more commonly represented by urinary albumin excretion more than 300mg/24h (3). This diabetic nephropathy is almost irreversible and ultimately leads to ESRD (1). An early manifestation of diabetic nephropathy is microalbuminuria, which is defined as elevated urinary albumin excretion below the level of clinical albuminuria (4) undetected by Albustix (Albustix-negative albuminuria). It can only be detected by special methods such as RIA, ELISA, and immunoturbidimetry (3). Diabetic nephropathy at this microalbuminuric stage is reversible with euglycaemic control. Therefore it is pertinent to detect nephropathy at or before microalbuminuric stage. The glomerular component first involved in diabetes is the basement membrane. The objective of this study was to ascertain if glomerular basement membrane involvement in diabetic patients could be detected by some anomalies in urine chemistry before microalbuminuria sets in. To this end we selected albustix negative diabetic patients and evaluated the urinary excretion of N-acetyl-beta-D-glucosaminidase (2-acetamidodeoxy-β-glucoside acetamidodeoxygluco hydrolase, EC 3.2.1.30) (NAG), and gamma-glutamyl transferase (g-glutamyl-peptide: amino acid γ-glutamyl transferase, EC 2.3.2.2) (GGT) to assess their significance as early markers of diabetic nephropathy). NAG is involved in mucopolysaccharide and glycoprotein metabolism in basement membrane (5). GGT is not involved in this metabolism and is located almost exclusively along the proximal tubular brush border (6, 7).

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MATERIALS AND METHODS

Known diabetic patients on anti diabetic drugs, reporting to Endocrinology OPD of Command Hospital (SC) Pune were screened for albuminuria. Albustix negative urine samples centrifuged at 2000 rpm for 10 minutes in sterile 15mL glass test tube, were used for quantitation of creatinine, albumin, NAG and GGT whereas corresponding urine samples in sterile plastic bottles were sent to Dept of Microbiology for culture to rule out urinary infection. The blood samples of these selected patients were assessed for glucose, urea and creatinine concurrently. Urinary analytes were analysed within 6 hours of voiding the samples.

Blood Glucose was estimated by GOD-POD method using Autopack reagents (Bayer India). Blood Urea was estimated by 'Marbach, Scott, Chawney and Fawcett method' based on Berthlot's reaction. Blood and urine Creatinine was estimated by fixed time kinetic method based on Jaffe's reaction.

Urinary albumin was estimated by immunoturbidimetric method using Boerhinger reagents (Germany) based on the following principle: Anti-human albumin antibodies react with the antigen human albumin in the sample to form antigen-antibody complexes which following agglutination is measured turbidimetrically.

Urinary enzyme NAG was estimated kinetically by using Sigma (USA) reagents (Junko et al). It is based on the principle that substrate 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG), hydrolyzed by the enzyme NAG at pH 5.7, liberates the chromogen 2-chloro-4-nitrophenyl (CNP) which absorbs light of 400nm. NAG activity is determined kinetically by calculating the rate of CNP formation in terms of change in absorbance at 400nm.

Urinary enzyme GGT was estimated kinetically by using Boerhinger (Germany) reagents (Persijn et al, J Clin Chem Clin Biochem 1976: 14:421). It is based on the principle that the enzyme GGT catalyses the transfer of glutamyl residue from L-γ- glutamyl-carboxy-3-nitro-4-anilide to the acceptor glycylglycine at pH 8.25. The released amino-5-nitro-2-benzoate absorbs light of 415nm. The increase in absorbance at this wavelength is directly proportional to the activity of GGT. The GGT activity is determined kinetically by calculating the rate of amino-5-nitro-2-benzoate formation in terms of change in absorbance at 415 nm.

All the three analytes albumin, NAG and GGT were analysed by using semi-autoanalyser Shimadzu CL-750 (micro-flow spectrophotometer) (8).

196 diabetics were included in this study as Patients and 48 healthy individuals as Normal Control. Urinary albumin was expressed as the ratio of milligrams of urinary albumin to grams of urine creatinine (Alb/Cre mg/g), while NAG and GGT were expressed as the enzyme activity per gram of urine creatinine (NAG/Cre U/g and GGT/Cre U/g respectively). The Upper Limit of Normal (ULN) was defined as Mean ± 2SD. Values above ULN were considered raised.

RESULTS

The mean age of Control and Patient group was 45.3 years and 54 years respectively. The duration of diabetes was in the range of 1 month to 40 years (95% patients in the range of 0.2-22 years).

The distribution pattern of urinary enzymes in control group and upper limit of normal (ULN) is given in TABLE 1. The urinary Albumin/Creatinine ratio, NAG/Creatinine ratio and GGT/Creatinine ratio in Control versus Patient are as per TABLE 2.

Diabetic patients were divided as: Normoalbuminuric with Alb/Cre ratio of < 30mg/g and Microalbuminuric with Alb/Cre ratio of > 30mg/g. The urinary Alb/Cre, NAG/Cre and GGT/Cre ratio in these 2 groups is as per TABLE 3. There was poor

TABLE 1
Distribution of Urinary Enzymes in Controls

Parameter	NAG/Cre ratio (U/g)	GGT/Cre ratio (U/g)
Mean	5.51	25.75
95% confidence interval	4.73-6.29	22.93-28.56
Mode	5.8	28.3
Median	5.55	26.2
95th percentile	10.53	40.53
Upper Limit of Normal (ULN)	10.67	44.35

Correlation between urinary albumin and urinary enzymes (Figure 1) and between urinary NAG and urinary GGT (Figure 2) in all diabetic patients. Correlation between urinary albumin and urinary enzymes in normoalbuminuric and microalbuminuric diabetic patients is as per Figure 3 and 4 respectively. No correlation was found between albumin and GGT in these groups of diabetic patients (r=0.08 and 0.06

TABLE 2
Urinary Albumin & Urinary Enzymes – Control Versus Patients

	CONTROL			PATIENTS		
Analyte	Range	Mean	SD	Range	Mean	SD
Alb/Cre (mg/g)	4.3-29.5	20.11	6.65	5.16-295.92	63.83	63.95 (p < 0.001)
NAG/Cre (U/g)	1.2-13.1	5.51	2.58	0.43-100.09	12.87	11.44 (p < 0.001)
GGT/Cre (U/g)	9.7-48.3	25.75	9.3	7.4 -103.97	35.43	16.79 (p < 0.01)

TABLE 3
Urinary Albumin and Urinary Enzymes in Normoalbuminuric and Microalbuminuric Diabetic Patients

	NORMOALBUMINURIC (n=94)			MICROALBUMINURIC (n=102)		
Analyte	Range	Mean	SD	Range	Mean	SD
Alb/Cre (mg/g)	5.16-29.56	23.04	5.48	33.38-295.92	101.41	69.95
NAG/Cre (U/g)	0.43-27.2	9.49	5.6	0.95-100.09	15.99	14.27
GGT/Cre (U/g)	7.4-62.2	31.51	14.5	8.10-103.97	39.04	18.00

TABLE 4
Excretion of NAG and GGT > ULN in Diabetic Patients

Diabetic Patients	Urinary NAG/Cre Ratio >ULN		Urinary GGT/ Cre Ratio >ULN	
	No. of cases	(%)	No. of cases	(%)
Normoalbuminuric (n=94)	32	34	6	6.4
Microalbuminuric (n=102)	62	63.7	25	24.5
Total (n=196)	94	49.5	31	15.8

respectively). In both these diabetic groups, no correlation was found between NAG and GGT.

Excretion of either urinary NAG or urinary GGT or both, more than ULN was present in 32 of 94 normoalbuminuric and 66 of 102 microalbuminuric diabetics (Table 4). The correlation between urinary albumin and urinary NAG in normoalbuminuric, microalbuminuric and overall diabetic patients with excretion of NAG more than ULN is as per Figure 5, 6 and 7 (r=0.312, 0.278 and 0.350; p<0.01) respectively.

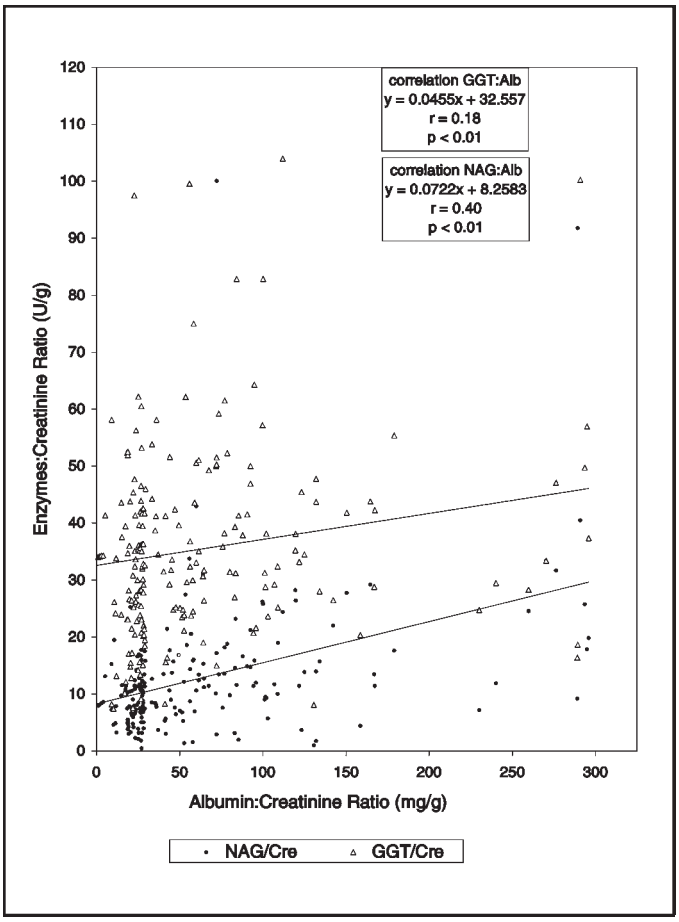

Figure 1. CORRELATION BETWEEN URINARY ALBUMIN AND ENZYMES IN DIABETIC PATIENTS (n=196)

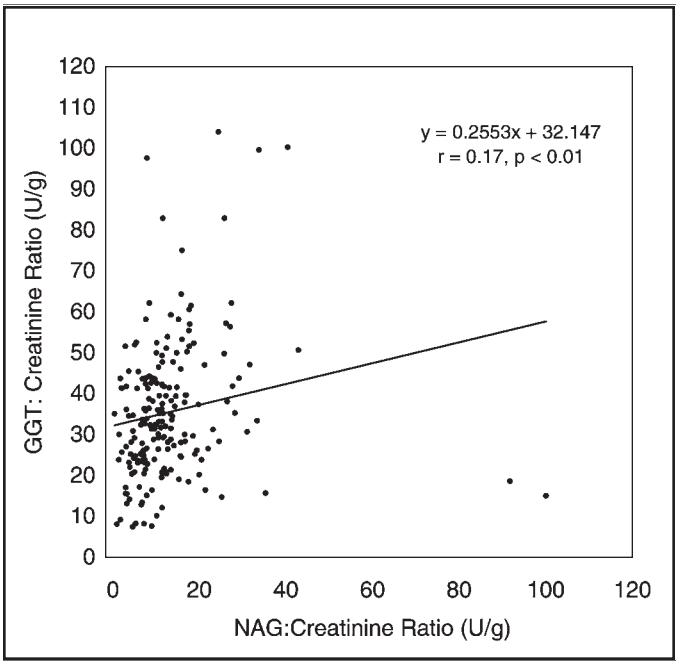

Figure 2. CORRELATION BETWEEN NAG & GGT IN DIABETIC PATIENTS (n=196)

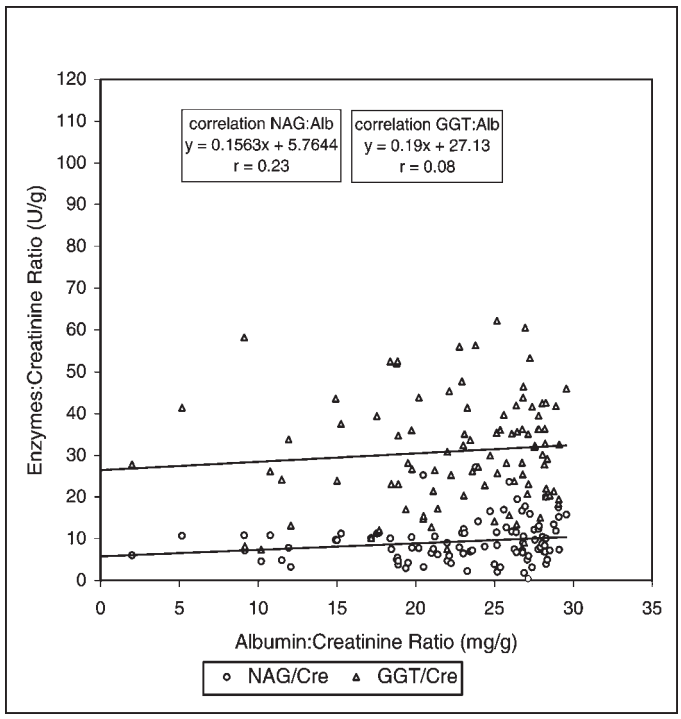

Figure 3. CORRELATION BETWEEN URINARY ALBUMIN AND ENZYMES IN NORMOALBUMINURIC DIABETIC PATIENTS (n=94)

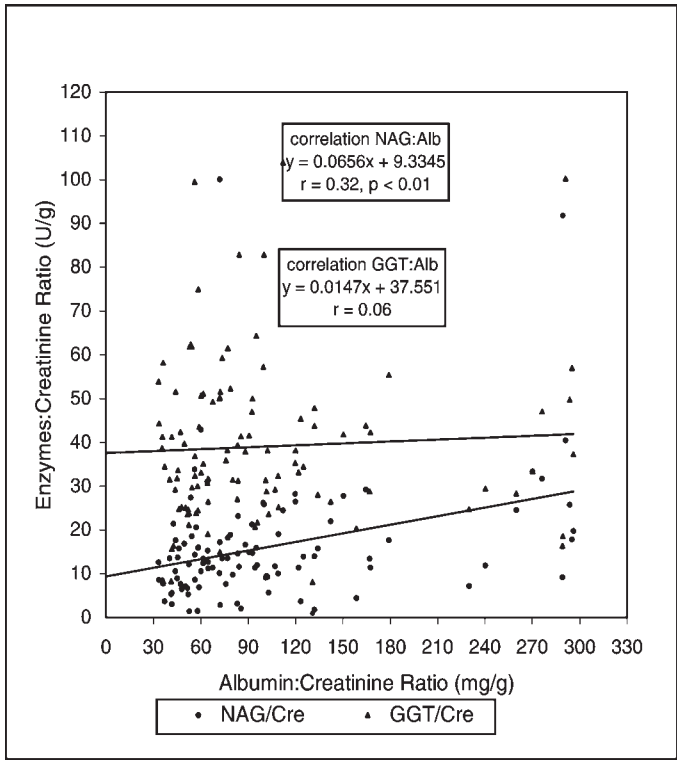

Figure 4. CORRELATION BETWEEN URINARY ALBUMIN AND ENZYMES IN MICROALBUMINURIC DIABETIC PATIENTS (n=102)

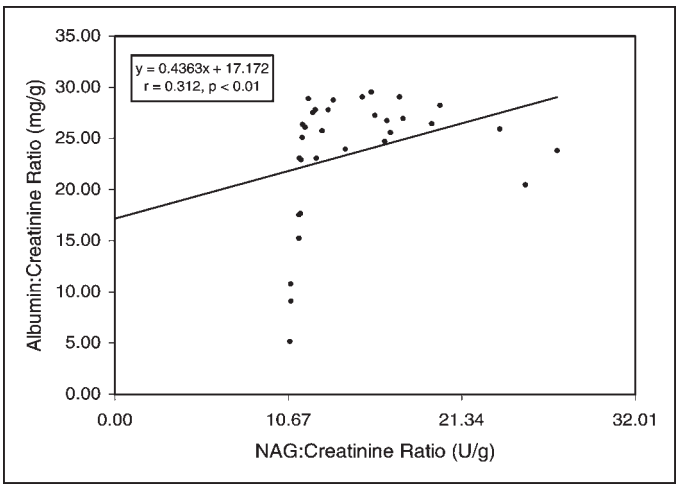

Figure 5. CORRELATION BETWEEN NAG AND ALBUMIN IN NORMOALBUMINURICS WITH NAG > ULN (n=32)

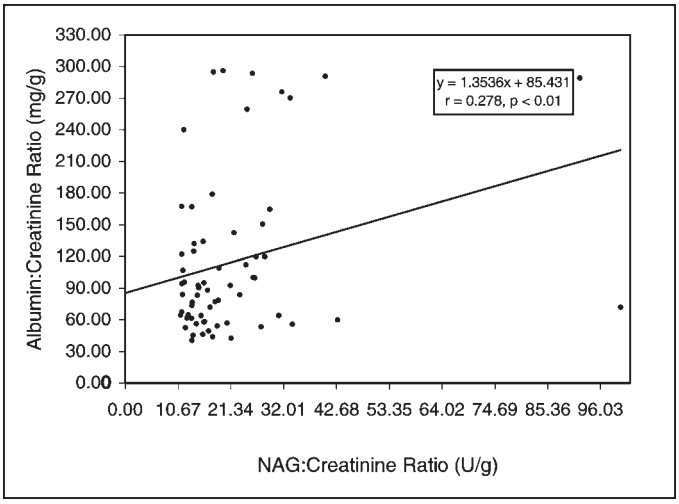

Figure 6. CORRELATION BETWEEN NAG AND ALBUMIN IN MICROALBUMINURICS WITH NAG > ULN (n=65)

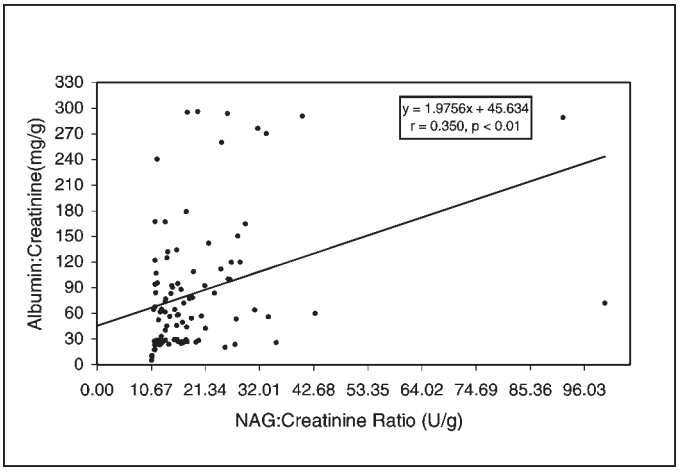

Figure 7. CORRELATION BETWEEN ALBUMIN AND NAG IN DIABETIC PATIENTS WITH NAG > ULN (n=97)

DISCUSSION

Diabetes mellitus is a chronic condition that poses a risk five times or even greater for developing nephropathy. It is known that microalbuminuria is the earliest manifestation of diabetic nephropathy and requires special assays. The glomerular component first involved in diabetes is the basement membrane, which might undergo a chemical rearrangement before proteinuria (microalbuminuria) sets in. In this study we have evaluated urinary enzyme NAG that is involved in mucopolysaccharides and glycoprotein metabolism and GGT, which is located almost exclusively along the tubular brush border of proximal tubule, as early markers of diabetic nephropathy.

The urinary albumin and urinary enzyme concentration was calculated in terms of their ratios with respect to creatinine concentration. The use of this ratio reduces the spread of data points by minimizing the variation due to diuresis induced concentration fluctuations (9, 10). In our study, the normal reference value (mean ± SD) of NAG excretion was 5.51 ± 2.58 U/g and GGT was 25.75 ± 9.3 U/g of creatinine (TABLE 1). This is in agreement with the reported normal values in terms of enzyme creatinine ratios determined by using random urine samples (10 -15). The excretion of NAG in diabetic patients was found increased to 12.87 ± 11.44 U/g creatinine as compared to 5.51 ± 2.58 U/g creatinine in healthy controls ($p < 0.001$) (TABLE 2). This is almost identical to the results of Nakamura S [11] who reported increased excretion of NAG, 10.3 ± 9.5U/g in diabetic patients (using random urines of 132 albustix negative diabetics) compared to 3.9 ± 2.1 U/g creatinine in healthy controls (using random urines of 59 normal subjects).

Correlation between the albumin creatinine ratio and enzyme creatinine ratio was analysed statistically using software EPI 2000. The urinary albumin was found to have some positive correlation with the urinary enzymes in overall diabetic patients ($r=0.18$, $p< 0.01$ for GGT and $r=0.4$, $p<0.01$ for NAG) (Figure 1). Minimal positive correlation ($r=0.17$, $p< 0.01$) was found between urinary NAG and urinary GGT (Figure 2). The correlation coefficient (r) of 0.4 ($p< 0.01$) between urinary enzyme NAG and urinary albumin in overall diabetic patients (n=196) is in agreement with the findings of Ellis et al [16] in which urinary NAG was found to positively correlate with urinary albumin creatinine ratio, $r=0.47$ ($p < 0.01$), on the basis of which he suggested urinary NAG as an early marker of diabetic nephropathy.

Diabetic patients were divided into two groups as normoalbuminuric and microalbuminuric. In both the groups, moderately positive correlation ($r=0.23$ & 0.32, $p < 0.01$) was found between urinary albumin and urinary enzyme NAG whereas no correlation between urinary albumin and urinary GGT (Figure 3, 4). The correlation with NAG was slightly better in normoalbuminuric patients having NAG excretion more than upper limit of normal ($r=0.31$), (Fig 5). This indicates the usefulness of urinary NAG over urinary GGT and suggests urinary NAG as better marker as predictor of diabetic nephropathy than urinary GGT. Thus our findings are in agreement with the reported data, which indicated that urinary GGT is not significant as early markers (17, 18) and there is no correlation between urinary NAG and urinary GGT (12, 19). However this finding is in contradiction to some reported data in which a significant decrease in urinary GGT excretion was reported in albuminuric diabetic patients (20). In our study urinary GGT excretion was found slightly more than the normal in both normoalbuminuric and microalbuminuric diabetic patients. The mean ± SD in control, normoalbuminuric and microalbuminuric groups was 25.75 ± 9.3, 31.51 ± 14.5 & 39.04 ± 18 U/g creatinine respectively ($p < 0.01$).

A moderate, positive correlation ($r=0.312$) was found between urinary albumin and NAG in normoalbuminuric cases with increased excretion of NAG (NAG creatinine ratio > ULN) (Figure 5). It was found that in normoalbuminuric cases, 34% have shown raised NAG excretion but GGT excretion was found raised only in 6.4% cases (TABLE 4). This result was found similar to the earlier published data (21) according to which GGT was found raised in 6.1% and NAG in 24.5% in normoalbuminuric diabetic patients. However in our study the increased excretion of NAG in 34% normoalbuminuric diabetic patients was considerably less than some of the other studies. Gibbs et al found increased excretion of NAG in 72% normoalbuminuric (22), Jones et al in 60% normoalbuminuric (23), Cohen et al in 92% diabetics without nephropathy (18) & Baggio et al in 85% normoalbuminuric (24). However in all these studies the sample size was small, number of diabetic patients in the range of 15-25 only. In our study not only the sample size was large (96 normoalbuminuric cases) but also the urinary albumin and urinary enzymes were estimated immediately within six hours of voiding the urine, thereby eliminating the effect of freezing, storage and preservatives.

When enzymuria in microalbuminuric diabetic patients was analysed NAG excretion was found raised in 63.7% whereas GGT excretion was found raised in 24.5% cases (Table 4). The increased excretion of NAG in 63.7% microalbuminuric diabetic patients was in agreement with the existing data (Jones et al) in which 66% microalbuminuric diabetic patients

were found to have increased NAG excretion (23). The correlation between urinary albumin and NAG in these cases (microalbuminuric with NAG >ULN) was also found moderately positive (r=0.278) and statistically significant (p<0.01) (Figure 6). No correlation was found between urinary albumin and GGT in microalbuminuric cases with GGT >ULN.

In overall cases 49.5% diabetic patients were found to have raised NAG level as compared to 15.8% with raised GGT levels. The correlation between urinary albumin and NAG was found positive with (r=0.350) (p<0.01), (Figure 7), whereas, no correlation was found between urinary albumin and GGT.

Urinary NAG, though better than GGT as marker of nephropathy, has poor correlation with urinary albumin excretion in diabetic patients, 'r' values ranging between 0.23 and 0.40 in various groups. At this low correlation coefficient, even though urinary NAG is elevated in diabetes mellitus, urinary NAG may not be a clinically useful predictor of diabetic nephropathy. Also no correlation was observed between duration of diabetes and any of the enzyme excretions. Therefore, in agreement with Agardh et al (25) who reported that Urinary NAG activity does not predict development of diabetic nephropathy and Mungan et al (26) who reported that there is no correlation between AER and urinary NAG activity and urinary NAG activity does not appear to be a useful marker for early detection of diabetic, we conclude that there is no clinical significance of urinary enzyme NAG or GGT or both as early markers of diabetic nephropathy.

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