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Variation of Prolidase Activity: A Predictor of Neuropathy due to Diabetes Mellitus?

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ABSTRACT
Prolidase is a member of the matrix metalloproteinase family responsible for collagen turnover which is implicated in the pathogenesis of diabetic neuropathy (DN). 50 (F-16, M-34) subjects suffering from T2DM, 46 subjects (F-21, M-25) with DN and 50 (F-19, M-31) healthy volunteers were selected to evaluate if there is any role of altered plasma prolidase activity in the development of diabetic neuropathy. HOMA-IR was applied for estimation of insulin resistance and NCV was performed to confirm the subjects of neuropathy. Estimation of prolidase activity was based on the principle proposed by Myara et al. (1984).No significant correlation between prolidase activity and HOMA-IR in the control group and group 1 categorized as patients of T2DM without any microvascular complications was found statistically. However in patients of T2DM with neuropathy (group 2) we found a significant correlation ($r=0.324$, $p=0.028$). Independent sample t-test between healthy control and group1 reveals statistically significant change of mean levels of HOMA IR ($t=7.174$, $p<0.001$) but not of prolidase ($t=1.174$, $p=0.243$) activity. Using t-test again between group 1 and 2 we found that the parameters HOMA-IR ($t=4.643$, $p<0.001$) and prolidase ($t=5.968$, $p<0.001$) were significantly higher in group 2 than in group 1. These findings imply a strong association between insulin resistance and increased collagen turnover in T2DM. ANOVA was performed and a strong statistical association was found in case of HOMA-IR ($F=50.266$, $p<0.001$) and prolidase activity ($F=31.006$, $p<0.001$) among different groups. These findings can hypothesize that collagen turnover and increased insulin resistance are possibly the underlying pathogenic mechanisms for the development of neuropathy caused by diabetes.

Keywords: *prolidase; collagen turnover; diabetes mellitus; diabetic neuropathy; homa ir.*

1. INTRODUCTION

Peripheral neuropathy in T2DM is characterised by distal symmetric polyneuropathy (DSP). This major complication of T2DM is multifactorial in origin [(1)]. Intensive glycemic control is insufficient to prevent either onset or progression of DSP and disease-modifying treatments for DSP have been disappointing. DNs are a major cause of disability and are also associated with reduced quality of life and increased mortality[(2)]. So, an effective management of hyperglycaemia, symptom control, prevention of foot ulcers and systemic infection through screening and surveillance remain mainstay of management of DN[(3)]. Performing an annual screening through a good neurological history, clinical examination by using sensitive screening tools can facilitate an early diagnosis. More sensitive and quantitative

measures of detecting early peripheral nerve injury including skin biopsy for intra-epidermal and dermal nerve fibre density and confocal corneal microscopy hold promise to identify neuropathy patients early in their disease course [4]. Most of the tests need expertise in related fields to carry them out. In the present study our aim is to find out a contributory biochemical change which can be easily detected in blood that will be helpful to the clinician and the diseased.

Prolidase (PLD, EC 3.4.13.9) is a member of the matrix metalloproteinase family [5,6]. It is the only human enzyme responsible for the digestion of iminodipeptides containing proline or hydroxyproline at their C-terminal end, being a key player in collagen turnover, extracellular matrix remodeling and cell growth [5,7-9]. Uzar et al. (2012) stated that there is increased plasma prolidase enzyme activity in neuropathy due to diabetes (DN) as evidence of increased collagen turnover [10].

Homeostatic model assessment- insulin resistance (HOMA IR) reflects insulin resistance and is associated with increased prevalence of impaired glucose tolerance (IGT) and type 2 diabetes in several populations [11-14].

We found insufficient and conflicting data regarding plasma prolidase activity, oxidative stress and their role in development of microvascular complications like neuropathy in patients with diabetes. [10,15] Moreover no data was found which can relate insulin resistance (IR), the main perpetrator of T2DM to prolidase activity. In this non-interventional hospital based case control study our aim is to evaluate plasma prolidase activity in the backdrop of insulin resistance.

2. METHODOLOGY

2.1 Study design:

The present study is a hospital based non-interventional case control study.

2.2 Study Population and Setting:

Patients attending the outpatient Department of Diabetology of our Institution for their routine follow up of T2DM which was diagnosed by the existing criteria for the disease by American Diabetes Association (ADA) (16-18).

2.3 Sample size:

Total of 146 subjects with possible matching were selected for the study. Out of them, 50 subjects (F-16, M-34) were patients of type-2 diabetes mellitus (T2DM) of ages between 36 and 65 (46.9 ± 6.03) years, 46 patients with DN (F-21, M-25) of ages between 45 and 64 (54.04 ± 4.85) years and 50 healthy volunteers (F-19, M-31) of ages between40 and 60years (48.2 ± 5.67) old were included in the study. Healthy volunteers and patients with T2DM without any micro-vascular complications were considered as control and Group 1 respectively. The other cluster namely diabetes with neuropathy was classified as Group 2 in the present study.

2.4 Selection of cases and controls: Inclusion and Exclusion Criteria: Cases of T2DM with neuropathy confirmed by abnormality in nerve conduction velocity- NCV were selected from the patients suffering from T2DM in the outpatient Department of Diabetology and two groups of controls (Control and Group: 1) were selected as follows:

1. Healthy subjects without any disorder that may lead to any derangement of our estimating parameters.
2. Recently diagnosed T2DM subjects without any micro-vascular complication after clinical examination (including ophthalmoscopy).

Voluntary participants for the healthy control group were selected after screening them for any existing metabolic disorder. Relatives of the patients were excluded during this selection. Patients suffering from major infections like tuberculosis, HIV, chronic heart failure, pregnant females, patients on antioxidant therapy, and patients with history of alcoholism, smoking and addiction to tobacco were excluded from our study. Past history of macroangiopathic complications of diabetes mellitus (like coronary artery disease, peripheral vascular disease and stroke) in group1 were excluded. Patients were

included provided they had no other metabolic, neoplastic, soft tissue (i.e. dermatological) or bone disorders that are established to alter prolidase activity and have attained the age of 18 years, so that they could give informed consent after understanding the objectives of the study.

Overall exclusion criteria for control group were same as those used for case selection procedure. As the participants attend the hospital OPD from a large rural base, they were expected to have approximately similar ethnicity, socioeconomic status and dietary habits.

The study followed the guidelines of the Helsinki declaration of 2009 [19] and was approved by the Institutional Ethics Committee. Informed consent was taken from every subject.

2.5 Collection and Storage of Samples:

12 hour fasting venous blood samples from all of the subjects (cases and controls) were collected in 3 vials (heparinised tube, fluoride-oxalate tube and plain tube). Blood in the fluoride-oxalate tube was used for the estimation of glucose. The heparinised sample was used for the estimation of plasma prolidase activity, while blood in plain tube was allowed to clot and serum was separated by centrifugation at 2500 rpm for 5 min at $4^{O}C$. Serum samples for the measurement of Insulin were stored at $-20^{O}C$ till estimation. Samples were thawed to room temperature before every assay, and repeated thaw was avoided.

3. ESTIMATION OF TEST PARAMETERS

3.1 Estimation of fasting blood glucose:

Quantitative estimation of blood glucose was done by Glucose oxidase / Peroxidase method [20] from the separated plasma by using the autoanalyzer ERBA XL 600. Internal quality control was performed simultaneously. All test reagents are supplied by Span Diagnostics Ltd. India and the quality control materials (Level 1 and 2) were supplied by Bio-Rad laboratories, USA.

3.2 Serum Insulin Estimation:

Serum insulin was assayed by ELISA kit by CALBIOTECH Inc. USA. This method has been reported to show a high degree of correlation with reference radioimmunoassay method. No cross reactivity with C-peptide was detected. The serum was separated at $4^{0}C$ and stored at $-20^{0}C$ till assay was done. Assays were done within one week from the date of collection in automated ELISA reader and washer from TECAN, Austria.

3.3 Computation of Insulin Resistance [21-23]:

Homoeostatic model for assessment (HOMA) of insulin resistance (HOMA IR) were calculated as percentage of a normal reference population of young people without diabetes mellitus. As a widely validated clinical and epidemiological tool for estimating insulin resistance and β cell function, the homeostasis model assessment (HOMA) is derived from a mathematical assess-ment of the balance between hepatic glucose output and insulin secretion from fasting levels of glucose and insulin [14,21,22]. This model requires only single measurement of insulin and glucose in the basal state and so, in some conditions, is a suitable alternative for large-scale epidemiologic studies to the sophisticated "gold standard" methods which usually require dynamic data via costly and invasive procedures. HOMA IR is computed with the formula: fasting plasma glucose (mmol/l) times fasting serum insulin (mIU/l) divided by 22.5 [14, 23].

3.4 Prolidase Activity Measurement: Adapted from method described by Myara et. al [24] This modification was done to reduce reagent consumption thus reducing the cost per test.

3.4.1 Principle:

Chinard's reagent (600mL of glacial acetic acid mixed with 400mL of 6 mol/L orthophosphoric acid and 25 g of ninhydrin was dissolved in the mixture at $70^{O}C$ temperature; for preparation of 6 mol/L orthophosphoric acid, 407mL of orthophosphoric acid (85%, d = 1.7) was added to 593mL of distilled water), standard proline solution (650 μmol/L in 0.45mol/L trichloroacetic acid), and 94 mmol/L Gly-l-Pro (Sigma Chemical Co.) was prepared and stored at 4°C. Plasma was diluted six-fold with buffer mixture containing

0.05 mmol/L Tris HCl buffer (pH 7.8) in 2 mmol of $MnCl_2$/L and was incubated for 24 hrs at 37°C. After incubation, prolidase reaction was initiated by adding 10 µL of the pre-incubated mixture to 10 µL of 94 mM Gly-l-Pro solution. After reaction initiation, it was incubated for 30 min at 37°C; reaction was stopped by adding 100 µL of 0.45 mol/L trichloroacetic acid. After that centrifugation at 2000 rpm for 5 minutes supernatant was separated from the small pellet at the bottom and 100 µL was used for proline estimation.

For measurement of enzymatic reaction, three tubes were selected and labelled as blank, standard and experimental. 250µl of glacial acetic acid and 250µl of Chinard's reagent were added in each tube. After this, 100µl of supernatant was added in experimental tube. 100µl of trichloroacetic acid (0.45mol/L) was added in blank tube and 100µl of standard proline solution was added in standard tube. All three tubes were incubated in water bath at 90^{O}C for 10 minutes and absorbance of each tube was taken at 515 nm. The amount of proline was determined by spectrophotometer at 515 nm.

3.4.2 Calculation of Prolidase enzyme activity:

For the estimation of prolidase we took 100µl from the total volume of 120µl. So, the ratio = 1.2. For the standard tube we took 100µl of the standard solution in µmol/L strength. So, the factor to determine the amount of proline in 100µl (finally in 600µl of standard tube) is=10^{-4}.

Hence, mmol of Proline formed during 30 minutes by 0.01ml of 6 times diluted plasma

$$= \frac{\text{Resultant Abs. of Test sample tube} \times \text{Conc. of Proline standard}}{\text{Resultant Abs. of Standard Tube}} \times 1.2 \times 10^{-4}$$

If we express the result per liter of undiluted plasma, during one minute, we obtain

$$\frac{\text{Resultant Abs. of Test sample tube} \times \text{Conc. of Proline standard}}{\text{Resultant Abs. of Standard Tube}} \times \frac{1.2 \times 10^{-4}}{30} \times 10^{5} \times 6$$

Moles of proline formed per liter of plasma per minute at 37^{O}C and pH 7.8. Simplifying the equation:

$$\frac{\text{Resultant Abs. of Test sample tube} \times \text{Conc. of Proline standard}}{\text{Resultant Abs. of Standard Tube}} \times 2.4 \text{ mmol/min/L}$$

STATISTICAL CALCULATIONS

Descriptive statistics of all the parameters including the age and sex distribution of each and every group was done. Pearson's bivariate correlation study was performed for any correlation between the parameters within a group. Student's't' test is used to compare means between two groups. The three groups were compared for intra and inter group differences among means of different test parameters by Analysis of Variance (ANOVA) tests. To check the statistical significance of nonparametric data like age distribution Chi-square test ($\chi 2$) was performed. Significance was considered at 95% confidence interval ($p<0.05$) for all statistical analysis. All statistical analyses was done using SPSS software version 16.0 for Windows.

OBSERVATIONS AND DISCUSSION

The aim of the study was to look into the association of neuropathy due to diabetes with plasma prolidase activity. For this, we had a study population of 146 subjects with 50 in the control group, 50 patients of T2DM without neuropathy and 46 patients of T2DM with neuropathy. The sex ratio between the groups are statistically insignificant ($\chi 2=1.893$, $p=0.388$). Thus the study population is sex matched. However age matching could not be done (ANOVA shows $F=22.182$ and $p=0.000$) because neuropathy development requires years following the onset of diabetes, this might be due to the complex anatomy of the peripheral and autonomic nervous systems, the multitude of pathogenic mechanisms involved and

the lack of uniformity of neuropathy measures as described by Ang et al (2014) [25].

In this study mean and SD of the HOMA IR and Prolidase Activity (mmol/L/min) are 1.96 ± 0.76 and 727.49 ± 200.03 respectively in the control group (Table: 1) . The values of the afore mentioned parameters in the same order for Group 1 and Group 2 are 12.08±9.95, 777.11±222.0 and 25.59±17.78, 1059.91±242.27 respectively (Table: 1).

Table: 1 Mean and SD of different studied parameters in our study groups:

	CONTROL (N=50)		GROUP: 1 (N=50)		GROUP: 2 (N=46)	
	MEAN	SD	MEAN	SD	MEAN	SD
FPG (mg/dl)	87.48	10.92	155.82	40.38	223.65	84.07
Fasting Insulin (µIU/ml)	9.17	3.73	29.67	21.51	45.38	21.64
HOMA IR	1.96	0.76	12.08	9.95	25.59	17.78
PROLIDASE Activity (mmol/L/min)	727.49	200.03	777.11	222.0	1059.91	242.27

Uzar et al (2012) and Sayin et al (2014) found relation between neuropathy due to diabetes and prolidase activity [10,15]. Sayin et al had performed a correlation study and got a negative correlation of prolidase with nitric oxide (NO) and MDA ($r = -0.911$, $p < 0.001$; $r = -0.905$, $p < 0.001$, respectively), while a positive correlation with total antioxidant status (TAS) ($r = 0.981$, $p < 0.001$) in DN patients. In our study population we applied Pearson's Bivariate Correlation but did not find any statistically significant correlation between prolidase activity with HOMA IR in the control group and group 1 (Table: 2). However in the Group 2 HOMA IR was found to be significantly correlated($r=0.324$, $p=0.028$) with prolidase activity (Table: 2).

Table: 2 Pearson's Bivariate Correlation between plasma prolidase activity and HOMA IR in our study groups:

CONTROL (N=50)		GROUP: 1 (N=50)		GROUP: 2 (N=46)	
'r'	'p'	'r'	'p'	'r'	'p'
0.007	0.960[a]	0.214	0.135[a]	0.324	0.028[b]

a-not significant statistically (>0.005)
b- significant statistically (<0.005)

In their study by Uzar et al (2012) [10] involving thirty-eight healthy participants, 40 patients with diabetes and without neuropathy, and 39 patients with neuropathy due to diabetes (DN) and had determined the activity of prolidase, levels of total oxidative status (TOS) and oxidative stress index (OSI) in the serum. They found the level of TAS was lower, while the levels of TOS, OSI and activity of prolidase were higher in both DN and control group with neuropathy due to diabetes, compared with the healthy subjects ($p < 0.05$). They had also pointed out that the prolidase activity is higher in the DN group than in the control group with diabetes ($p = 0.001$). On the contrary Sayin et al (2014) [15] were foremost in showing the decreased serum prolidase enzyme activity in DN than the control group ($p = 0.003$). They had enrolled forty-five patients with DN and 40 healthy controls. They had in addition mentioned about a negative correlation of prolidase activity and MDA level ($r = -0.905$, $p <0.001$). Decreased collagen turnover in DN patients was probably the reason for this finding. Independent sample t-test between the healthy control and diabetes without neuropathy group of our study population reveals statistically significant increased levels of HOMA IR, in the later group than the preceding one $t=7.74$, $p=0.000$ (Table: 3). Even though we find a higher value of mean prolidase activity than the control group but this is statistically found to be insignificant $t=1.174$ and $p=0.243$ (Table: 3).

Table: 3 Independent sample t-Test of different parameters between our study Groups:

Between Control (N=50) and Group: 1 (N=50)		
	't' value	'p' value
HOMA IR	7.174	=0.000[b]
PROLIDASE	1.174	0.243[a]
Between Group: 1 (N=50) and Group: 2 (N=46)		
HOMA IR	4.643	=0.000[b]
PROLIDASE	5.968	=0.000[b]

a-not significant statistically (>0.005)
b-highly significant statistically (<0.001)

These findings indicate the absence of collagen turnover in the recently diagnosed subjects of T2DM without neuropathy as reflected by plasma prolidase activity. On performing Independent sample t-test between diabetes with and without neuropathy group we found that HOMA IR, and prolidase levels are significantly higher in diabetes with neuropathy group than the other groups (t=4.643, p=0.000; and t=5.968, p=0.000 respectively) (Table: 3). The statistical significance in case of HOMA IR and prolidase is stronger (at 0.001 levels) (Table: 3). These findings implies a strong association between uncontrolled blood sugar level due to insulin resistance (reflected by increased HOMA IR), increased collagen turnover (reflected by increased prolidase activity) related pathology in the subjects suffering from neuropathy with diabetes than the subjects of diabetes without any complication.

We also performed the analysis of variance test (ANOVA) to compare the intergroup mean deviations from each other among the three mentioned group (Table: 4).

Table: 4 Analysis of Variance (ANOVA) between different parameters of three groups:

Parameters	'F' value	'p' value
HOMA IR	50.266	=0.000[a]
PROLIDASE	31.006	=0.000[a]

a- highly significant statistically (<0.001)

These findings (HOMA IR: F=50.266, p=0.000; and Prolidase activity: F=31.006, p=0.000) (Table: 4) also strongly suggest the increased statistical association of afore mentioned mechanisms for the development of neuropathy due to diabetes. However this study did not consider the presence of interfering basic amino acids lysine, hydroxylysine and ornithine in the plasma. Food habits, life style, body mass index (BMI) and lipid profile parameters those can contribute to the development of HOMA IR were not taken into consideration.

CONCLUSION

The association derived from this study indicates that collagen turnover and increased insulin resistance being contributory towards development of diabetic neuropathy. Prospective studies with large sample population are necessary to confirm the findings of this study for better understanding of the pathogenesis of this condition to open up more avenues for study, treatment and prevention of neuropathy due to T2DM.

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