6.1. INTRODUCTION

Patients with Type 2 diabetes mellitus share a pathophysiology that involves the pancreatic β-cells, the liver, and peripheral target tissues, namely skeletal muscle and adipose tissue. A variable degree of β-cell dysfunction is present in these patients in addition to hepatic insulin resistance, resulting in glucose overproduction. Glucose uptake in muscle cells requires insulin binding to cell-surface receptors and activation of the insulin signaling cascade, which in turn facilitates the movement of glucose across the cell membrane, ultimately the glucose is stored as glycogen or is used as an energy source via glycolysis to lactate or mitochondrial oxidation. Glucose metabolism can affect a wide variety of cellular functions and signaling pathways. Chronic hyperglycemia has been firmly established as a key factor in the development of diabetic microvascular disease affecting the retina, renal glomerulus and vasa nervorum. Most patients with type 2 diabetes are diagnosed in the relatively late stages of a long and complex pathological process. The pathogenic process of diabetes has its origin in the patients’ genotype, and may be influenced by intrauterine experience, before being moulded throughout life by environmental factors. Recent research has provided unequivocal evidence that improved control of blood glucose and vascular risk factors will delay the onset and reduce the severity of diabetic complications.

6.2. OBJECTIVES OF THE STUDY

a) To study the 2245 G/A and G82S polymorphism of RAGE gene.

b) To estimate the level of arsenic on Diabetic cases.

c) To study the blood level of aldose reductase, SOD & glutathione peroxidase.
6.3. STUDY GROUP

A case control study was conducted on all cases with Diabetes Neuropathy and Diabetes Retinopathy who were referred to the Endocrine department of Ramakrishna Mission Seva Pratishthan, Kolkata, India, from November 2012 to June 2015. A total of 112 DN patients and 28 DR patients were included. 30 age sex matched control cases were recruited as healthy human being from same Health Institute.

6.4. DESCRIPTION OF EXPERIMENTS

6.4.1. DETAILED HISTORY TAKEN FROM STUDY GROUP

A detailed structured questionnaire was designed which covers general information on socio-demographic, age, family history of patients, general medical history, types of drug used, different bio-chemical parameters like blood sugar level, glycated haemoglobin (HbA1c), Urea ,Creatinine, lipid profile etc. and occupational history. All the records were filled up by an interview with patients.

6.4.2. COLLECTION OF BLOOD SAMPLE

5ml peripheral blood was collected in EDTA vial.

6.4.3. MOLECULAR STUDY OF RAGE GENE

Genomic DNA was extracted from each sample of blood using a commercial kit (QIAGEN kit). PCR was performed from the isolated DNA.

6.4.3.1. 2245 G/A POLYMORPHISM STUDY

This study was done by the method based on Kankova K et al 2001. In this study, a two-step nested PCR was used since this polymorphism lies in a highly homologous region. In silico PCR
was applied in this study to make sure the primers were targeted to the correct loci prior to the actual PCR. In the first PCR,

Forward primer : 5’ GCC CCA TTC TGG CCT TAT CCC TAA 3’
Reverse primer : 5’ CCA CCA TGC CTG GCT AAT TTT GT 3’

PCR amplification were performed to amplify a 294 bp product in a total reaction volume of 26 µl containing 50 ng of DNA, 200nM each of dATP,dTTP,dCTP and dGTP, 12.5 pmol each of the primers, 1U AmpliTaq Gold DNA Polymerase, and 1X buffer (1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, pH 8.3). The mixture was then subjected to initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94 º C for 15 sec, followed by annealing at 60 º C for 30 sec and elongation at 70º C for 45 sec and a final extension at 72 ° C for 8 min in a programmable thermal cycler (model 2720, Applied Biosystem). A sample of the amplified product (10 µl) was diluted with 500 µl of sterile distilled water and 1.5 µl was used for the second PCR.

The second PCR was performed using the following primers

Forward primer : 5’ ACA CTT TGG GAG GCT GCT GC 3’
Reverse primer : 5’ CCA CCA TGC CTG GCT AAT TTT GT 3’

The second PCR were performed to amplify a 116 bp product. All the products were used in the same quantity and concentration. The PCR amplicon was subjected to an initial denaturation (95 ° C for 3 min), followed by amplification comprising of 40 cycles, with each cycle consisting of a sequence of temperatures set at 94 ° C for 10 sec(denaturation), 56.7 ° C for 20 sec (annealing) and 72 ° C for 20 sec(elongation) , followed by a final extension at 72 ° C for 8 min.
Restriction endonuclease digestion: The change of nucleotide (guanine to adenine) at intron 8 region creates a PstI restriction site (CTGCA/G). Restriction analysis was performed with PCR products digested overnight with 5U of restriction nuclease PstI (Thermo Scientific, Lithuania) at 37 ºC. The digested products were immediately separated by electrophoresis in 6% (w/v) agarose gel with ethidium bromide and visualised under UV. Subsequent digestion with PstI revealed fragments of 95 bp and 21 bp for the mutated minor allele 2245A. The wild-type major allele 2245G was 116 bp long since it does not carry the restriction site.

6.4.3.2. GLY 82 SER POLYMORPHISM STUDY

A PCR-RFLP assay was used to determine the RAGE Gly82Ser polymorphism by the method based on Kanakova et al., 1999. PCR was done in 26µL reaction mixtures containing 200 ng of DNA, 200nM each of dATP, dTTP, dCTP and dGTP, 0.25 µmol/L each of the primers, 1U AmpliTaq Gold DNA Polymerase, and 1X buffer (1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, pH 8.3). The primers used in this reaction were:

Forward Primer: 5´-GTAAGCGGGGCTCCTGTTGCA-3´;
Reverse Primer: 5´-GGCCAAGGCTGGGGTTGAAGG-3´

PCR amplification were performed to amplify a 397 bp product. The PCR was subjected to an initial denaturation (95 º C for 5 min), followed by amplification comprising of 35 cycles, with each cycle consisting of a sequence of temperatures set at 94 º C for 30 s (denaturation), 62 º C for 40 sec (annealing) and 72 º C for 45 sec (elongation), followed by a final extension at 72 º C for 10 min.

The 397-bp PCR products were digested by the restriction enzyme AluI (Thermo Scientific, Lithuania), 5 units for 16 h at 37 ºC, followed by electrophoresis on a 2% agarose gel.
The digestion revealed fragments of length 249, 123, and 26 bp for the wild-type Gly82 allele and 181, 123, 67 and 26 bp for the variant Ser82 allele.

### 6.4.4. STUDY OF ENVIRONMENTAL FACTOR

#### 6.4.4.1. COLLECTION OF HAIR SAMPLE

~ 500 mg hair sample was collected for each patient from behind the ear close to the scalp with a diameter of about 1 cm.

#### 6.4.4.2. ESTIMATION OF ARSENIC

Hair samples was digested with 5ml of concentrated Nitric Acid & 3ml of concentrated sulfuric acid following the method of Agahian et al 1990. Flow-injection-hybride generation-atomic absorption spectrometry (FI-HG-AAS) was used for estimation of arsenic in the collected bio-samples.

### 6.4.5. STUDY OF ENZYMES

#### 6.4.5.1. ESTIMATION OF ALDOSE REDUCTASE ACTIVITY

Blood was drawn from the subjects into EDTA anticoagulant tubes and immediately transported to the laboratory on ice. Red blood cells (RBC) were separated by centrifugation, washed thrice with saline, and stored at – 20 °C until further analysis.

A 10% erythrocyte suspension was made by adding 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. The suspension was lysed by repeated freezing and thawing (three cycles) and centrifuged to remove insoluble material, if any. ALR2 activity was measured spectrophotometrically using an appropriately diluted hemolysate according to method of Suryanarayana P et al,2004 using a SpectraMax spectrophotometer (SPECORD 50 PLUS). One unit was defined as micromoles NADPH oxidized/g Hb/ min. The assay mixture in 1 ml
contained 50 μmol potassium phosphate buffer pH 6.2, 0.4 mmol lithium sulfate, 5 μmol 2-mercapto ethanol, 10 μmol DL-glyceraldehyde, 0.1 μmol NADPH and enzyme preparation (hemolysate). The assay mixture was incubated at 37 °C and initiated by the addition of NADPH at 37 °C. The change in the absorbance at 340 nm due to NADPH oxidation was estimated.

6.4.5.2. ESTIMATION OF SUPEROXIDE DISMUTASE

This method based on Woolliams et al.(1983) employs Xanthine and Xanthine Oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The Superoxide Dismutase(SOD) activity was then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of I.N.T. under the assay condition. Quantitative in vitro determination of SOD activities in whole blood were estimated with 0.5ml heparinized whole blood using Ransod Superoxide Dismutase assay kit (Randox, United Kingdom). The whole blood was centrifuged at 3000 rpm for 10 minutes followed by aspiration of plasma to obtain the erythrocytes which were washed with 0.9% NaCl solution. The erythrocytes were made upto 2 ml with cold redistilled water followed by incubation at 4ºC. The lysate was assayed by UV-Visible Spectrophotometer (SPECORD 50 PLUS ) at the wavelength of 505 nm. Appropriate negative and positive control were maintained with each batch of estimation.

6.4.5.3. ESTIMATION OF GLUTATHIONE PEROXIDASE

This method was based on Paglia and Valentine(1967). Glutathione Peroxidase catalyzes the Oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance
at 340nm was measured. Quantitative *in vitro* determination of glutathione peroxidase activities in whole blood were estimated with 0.05 ml whole blood using Ransel Glutathione Peroxidase assay kit (Randox, United Kingdom). The samples were assayed by UV-Visible Spectrophotometer (SPECORD 50 PLUS) at a wavelength of 340nm. Appropriate negative and positive control were maintained with each batch of estimation.

**6.4.6. STATISTICAL ANALYSIS**

An exhaustive search of various subsets of explanatory variables (age, sex, duration of disease, area, educational status, monthly income, BMI, fasting blood sugar, post prandial blood sugar, HbA1c, urea, creatinine, triglyceride, cholesterol) was carried out for the cases corresponding to with Diabetes and control. The logistic regression analysis was performed by SPSS statistical package. Inter group comparisons for risk factors were performed using analysis of variance (ANOVA). Student’s *t* test was also used for other parameters. The value of *p*<0.05 was considered as statistically significant.

**6.5. OBSERVATION OF THE STUDY GROUP**

**6.5.1. DETAILED HISTORY OF THE STUDY GROUP**

Out of 140 diabetic patients, 112 had diabetic neuropathy and 28 patients had diabetic retinopathy. 30 cases were selected as healthy control.

Among the total number of individuals studied, number of male cases were more than female ones. In healthy control, number of male cases were 18 and number of female cases were 12. In neuropathy cases number of male patients were 69 whereas number of female patients were 43. In retinopathy cases also, male cases were higher in number (16) than female cases (12).
As our study centre was located in Kolkata so most of the patients were from there. More than 50% of cases were from Kolkata. In healthy control, neuropathy and retinopathy cases the number of patients from Kolkata were 16, 46 and 17 respectively.

After comparing the occupational details between healthy control and diabetic patients it had been found that most of the cases had a sedentary lifestyle. 46.67%, 41.07% and 28.57% cases in healthy control, neuropathy and retinopathy respectively were in service as their occupation.

Most of the cases, neuropathy (47.32%) and retinopathy (57.14%), were of high school educated.

In control cases, 33.33% came from per month income group >Rs. 15,000. In neuropathy cases, most of them (43.75%) came from an average per month income of Rs.11000-15000. But in retinopathy cases most of them (42.86%) were with no income group (Retired persons were included in this group). So they were reported at late stage of the disease.

Our data showed that in neuropathy cases greater percentage of cases (33.33% in male cases and 44.19% in female cases) lied between the duration limit of 6-10 years. Where in other hand in retinopathy patients with highest percentage (50% in male cases and 41.67% in female cases) had a duration greater than 15 years.

According to our data, hypertension was the most commonly found complication of diabetes.

Our data suggested that maximum number of male cases (50.72%) and female cases (48.84%) had a highest prevalence of sensory-motor polyneuropathy.

Our study had shown that BMI is low in patients than healthy control. The average BMI of control cases were 25.81±1.0. In neuropathy cases the average BMI was 22.50± 0.28 (p<0.001) and in retinopathy cases the value was 22.06±0.49 (p<0.05). Both were statistically significant.
6.5.2. BIOCHEMICAL FACTORS

6.5.2.1. BLOOD SUGAR PROFILE

Blood sugar levels were higher in diabetic cases than control cases. All diabetic parameters like fasting, post pandial and HbA1C were elevated in an significant amount. In neuropathy cases fasting, post pandial and HbA1C levels were 166.92±9.41 mg/dL, 275.20±15.94 mg/dL and 8.78± 0.22 % respectively. In retinopathy cases values were 219.25±21.66 mg/dL, 313.88±37.1 mg/dL and 9.79±0.51 %.

6.5.2.2. KIDNEY PROFILE

Both neuropathy and retinopathy cases had higher value of urea and creatinine than healthy control. On average 41.87±3.1 mg/dL and 39.75±3.08 mg/dL urea level were found in neuropathy and retinopathy cases respectively which were significantly(p<0.001) higher than healthy cases (22±2.3 mg/dL). The average amount of creatinine present in neuropathy and retinopathy cases were 1.10±0.06 mg/dL and 1.26±0.17 mg/dL respectively which was higher than normal 0.9± 0.03 mg/dL.

6.5.2.3. LIPID PROFILE

Higher values of Triglyceride and Cholesterol was found in neuropathy and retinopathy patients. Triglyceride level were significantly (p<0.001) elevated in both cases (in neuropathy 144.20±21.5 mg/dL and in retinopathy 158.24±25.66 mg/dL) than normal (89±8.01 mg/dL). Average value of Cholesterol also significantly (p<0.001) increased in neuropathy cases (172.84±9.94mg/dL) as well as retinopathy cases (184±13.98mg/dL) than healthy control (112±13.32 mg/dL).
6.5.3. MOLECULAR STUDY

6.5.3.1. 2245 G/A POLYMORPHISM

2245 G/A polymorphism were detected by PCR-RFLP method. Polymorphism was tested by specific bands after agarose gel electrophoresis. Comparing neuropathy and retinopathy, higher percentage (75%) of Polymorphism was present in retinopathy than neuropathy. HbA1C is an average status of blood sugar control. The higher values indicates poorer control. In our data it has been shown that more polymorphism were found in cases with poorer control of disease. 87.50% polymorphism were found in Neuropathy cases with HbA1C range between 9.6-10.5 and 72.73% polymorphism were found in Retinopathy cases with HbA1C range between 9.6-10.5.

6.5.3.2. Gly82Ser POLYMORPHISM

Another polymorphism Gly82Ser was tested in our study. Our data showed that retinopathy patients (with 66.67%) were more prone to G82S polymorphism than neuropathy. In our study we had found that, 73.08% polymorphism were in Neuropathy cases with HbA1C range between 8.6-9.5 and 88.89% polymorphism were in Retinopathy cases with HbA1C range between 8.6-9.5. So we can conclude uncontrolled blood sugar level had effect on the occurrence of the polymorphism.

6.5.4. ENVIRONMENTAL FACTOR

In our study, patients had significantly high (p<0.05) level of arsenic in both neuropathy (1.96±0.28 mg/kg) and retinopathy (2.68±0.565 mg/kg) cases as compared to healthy control (0.18±0.07 mg/kg). When arsenic level were compared to different type of neuropathy cases in
our study, it had been found that the mean arsenic level were highest (2.87±1.22 mg/kg) in the early polyneuropathy cases.

6.5.5. STUDY OF ENZYMES

6.5.5.1. ALDOSE REDUCTASE ACTIVITY IN THE STUDIED CASES

Our data showed that aldose reductase activity was higher (3.89 units/g Hb in neuropathy and 3.79 units/g Hb in retinopathy) than healthy individuals (1.80 units/g Hb). No significant difference was found between aldose reductase values of those complications. In comparison between different type of neuropathy cases with respect to aldose reductase activity, the activity of this enzyme was highest (5.63±3.17 units/g Hb) in early polyneuropathy amongst all type neuropathy cases.

6.5.5.2. SUPER OXIDE DISMUTASE ACTIVITY IN THE STUDIED CASE

In this study, we had found that value of SOD was much higher in patients than healthy subjects. In neuropathy cases the mean SOD value was 186.5±13.69 U/g Hb whereas in retinopathy cases the mean SOD value was 284.34±63.82 U/g Hb. Both the values had high significance level (p<0.001) than control cases (76±9.01 U/g Hb). When these SOD values were compared between different type of neuropathy cases in our study, it showed that the mean SOD value was highest in sensory motor polyneuropathy cases among all type of neuropathy cases.

6.5.5.3. GLUTATHIONE PEROXIDASE ACTIVITY IN THE STUDIED CASE

A comparative study was done between healthy individuals and patients with neuropathy and retinopathy with respect to the Glutathione Peroxidase value. Patients with neuropathy (155.97±13.67 U/g Hb) and retinopathy (132.88±26.34 U/g Hb) had much higher value than healthy controls (88.19±12.37U/g Hb). GPx values were also compared between different type
of neuropathy cases found in our study. The result showed that the GPx activity was highest in sensory polyneuropathy cases.

**6.6. CONCLUSION**

- Incidence of T2DM is on the rise in West Bengal.
- Out of 112 Neuropathy cases 61.61% patients were male and in Retinopathy cases out of 28 cases, 57.14% cases were male.
- Male and female ratio was 1.5:1 on average in our study.
- In Neuropathy, 33.33% of patients were above 50 years of age while in Retinopathy 43.75% patients were above the age group of 50 years. It indicates that retinopathy is more prone in a late stage.
- In our study more than 50% of cases were from Kolkata (urban area).
- After comparing the occupational details between healthy control and diabetic patients it had been found that most of the cases had a sedentary lifestyle.
- In neuropathy 47.32% and in retinopathy 57.14% cases were of high school educated.
- When we compared individual income per month between healthy control and diabetes patient, most of them (43.75%) in neuropathy cases came from an average income of Rs.11000-15000. But in retinopathy cases most of them (42.86%) were with no income group as because retired persons were included in this group.
- Our data showed that in neuropathy cases greater percentage of cases (33.33% in male cases and 44.19% in female cases) lied between the duration limit of 6-10 years and in retinopathy patients, highest percentage (50% in male cases and 41.67% in female cases) had a duration greater than 15 years.
• Hypertension was the most commonest complication of diabetes cases both in male and female ones.

• 50.72% male cases and 48.84% female cases had a prevalence of sensory-motor polyneuropathy, the highest amongst all.

• Our study had shown that BMI was low in patients than healthy control. This is an important indicator of poor health status.

• The average BMI of control cases were 25.81±1.0. In neuropathy cases the average BMI was 22.50±0.28 (p<0.001) and in retinopathy cases the value was 22.06±0.49 (p<0.05). Both were statistically significant.

• All diabetic parameters like fasting, post prandial and HbA1C were elevated in an significant amount. All these values were statistically (p<0.001) significant.

• On average 41.87±3.1 mg/dL and 39.75±3.08 mg/dL urea level were found in neuropathy and retinopathy cases respectively which were significantly (p<0.001) higher than healthy cases (22±2.3 mg/dL).

• The average amount of creatinine present in neuropathy and retinopathy cases were 1.10±0.06 mg/dL and 1.26±0.17 mg/dL respectively which was higher than normal 0.9±0.03 mg/dL.

• Triglyceride and Cholesterol values in neuropathy and retinopathy patients were higher than normal.

• Triglyceride level were significantly (p<0.001) elevated in both cases ( in neuropathy 144.20±21.5 mg/dL and in retinopathy 158.24±25.66 mg/dL) than normal (89±8.01 mg/dL).
Summary

- Average value of Cholesterol also significantly (p<0.001) increased in neuropathy cases (172.84±9.94mg/dL) as well as retinopathy cases (184±13.98mg/dL) than healthy control (112±13.32 mg/dL).
- Comparing 2245 G/A polymorphism in neuropathy and retinopathy cases, higher percentage (75%) of Polymorphism was present in retinopathy than neuropathy.
- 87.50% 2245 G/A polymorphism were found in Neuropathy cases with HbA1C range between 9.6-10.5.
- 72.73% polymorphism were found in Retinopathy cases with HbA1C range between 9.6-10.5.
- Our data showed that retinopathy patients (with 66.67%) were more prone to G82S polymorphism than neuropathy.
- 73.08 % Gly82Ser polymorphism were found in Neuropathy cases with HbA1C range between 8.6-9.5
- 88.89 % polymorphism were found in Retinopathy cases with HbA1C range between 8.6-9.5.
- Patients had significantly high (p<0.05) level of arsenic in both neuropathy (1.96±0.28 mg/kg) and retinopathy (2.68±0.565 mg/kg) cases as compared to healthy control (0.18±0.07 mg/kg).
- In early polyneuropathy, mean arsenic level was higher than other cases.
- Our data showed that aldose reductase activity was significantly higher (3.89 units/g Hb in neuropathy and 3.79 units/g Hb in retinopathy) than healthy individuals (1.80 units/g Hb).
- It was found that the activity of aldose reductase of early polyneuropathy cases was highest among all type neuropathy cases.
• In neuropathy cases the mean SOD value was 186.5±13.69 U/g Hb whereas in retinopathy cases the mean SOD value was 284.34±63.82 U/g Hb.

• When comparing with healthy control, both the values had high significance level (p<0.001) than control cases (76±9.01 U/g Hb).

• The level of super oxide dismutase in sensory motor polyneuropathy cases was highest among all type neuropathy cases.

• When we compared Glutathione Peroxidase value between healthy individuals and patients with neuropathy and retinopathy, patients with neuropathy (155.97±13.67 U/g Hb) and retinopathy (132.88±26.34 U/g Hb) had much higher value than healthy controls (88.19±12.37U/g Hb).

• When we compared GPx values in all type of neuropathy cases, it was found that the Glutathione Peroxidase activity of sensory polyneuropathy cases was highest among all type of neuropathy cases.
STUDY OF PREDISPOISING FACTORS AND ENVIRONMENTAL EFFECTS ON DIABETES MELLITUS

Population Study
- Age
- Gender
- Social Status

Biochemical Factors
- FBS, PPBS, HbA1c, Urea, Creatinine, Lipid Profile, Kidney

Molecular Study
- 2245 G/A polymorphism
- G82S polymorphism

Environmental Study
- Arsenic Estimation

Enzyme Estimation
- Aldose reductase
- SOD
- GPx

Methods followed:
- FBS, PPBS by GOD-POD
- HbA1c by HPLC
- Urea by Berthelot
- Creatinine by Jaffe’s
- Lipid profile by GPO-PAP

PCR followed by RFLP
- 2245 G/A by Kankova K et al, 2001
- G82S by Kankova et al, 1999

By Agahian et al, 1990 method

Data collection from questionnaire

Statistical Analysis were performed using
- Student’s t-test
- Logistic Regression Analysis

DN: Male cases > Female cases
DR: Male cases > Female cases
Duration: Avg 6-10 yrs for DN
Avg >15 yrs for DR

FBS: DN↑ DR↑
PPBS: DN↑ DR↑
HbA1c: DN↑ DR↑
Urea: DN↑ DR↑
Creatinine: DN(N) DR↑
Lipid profile: DN↑ DR↑

2245G/A: Male-Healthy (27.77%), DN (46.38%), DR(43.75%)
Female-Healthy (16.67%), DN (44.19%), DR(75%)
G82S: Male-Healthy (22.22%), DN (39.13%), DR(50%)
Female-Healthy (8.33%), DN (53.49%), DR (66.67%)

Arsenic: Male cases- DN↑ DR↑
Female cases- DN & DN not significantly higher

Aldose reductase- DN↑ DR↑
SOD- DN↑ DR↑
GPx- DN↑ DR not significantly higher