"Biochemical studies and genetic polymorphism involved in Diabetes in North Indian Population"

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Dedication

To

The Spirit of my Parent

DECLARATION

I, GUNJAN MISRA, hereby declare that the research work embodied in this Ph.D thesis titled "Biochemical studies and genetic polymorphism involved in Diabetes in North Indian Population" has been carried out by me under the supervision of Prof. M.Y.Khan, Professor, Department of Biotechnology, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University) Lucknow and the co-supervision of Dr. Varsha Gupta, Assistant Professor, Department of Biotechnology, Institute for Biosciences and Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur. This research work is an original work and it has not been previously submitted in part or full for any other degree or diploma in this University or any other University. All the above given information is true to the best of my knowledge.

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CERTIFICATE

This is to certify that the thesis titled "**Biochemical studies and genetic polymorphism involved in Diabetes in North Indian Population**" submitted by Ms. **GUNJAN MISRA** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) Regulation 1999 as amended in 2008/2010 /2013 and it is fit for submission and evaluation for the award of the Degree of Doctor of Philosophy of the University.

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ABBREVIATIONS

BMI	Body Mass index
BP	Blood Pressure
c-DNA	Complementry Deoxy Nucleic Acid
Cu	Copper
CVD	Cardiovascular Disease
Cm	Centimeter
˚C	Degree centigrade
DAS	Disease Activity Score
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
Hb	Haemoglobulin
HCl	Hydro Chloric Acid
HDL	High Density Cholesterol
IU	International Unit
IL	Interleukin
Kg	Kilogram
L	Litre
LYP	Lymphoid Protein Tyrosine Phosphate
LDL	Low Density Cholesterol
m-RNA	Messenger Ribo Nucleic Acid
µ	Micro
ml	Milli litre
MDA	Malondialdehyde
MQ	Milli Que
M	Molar
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NADPH	Nicotinamide Adenine Di Nucleotide Phosphate
OD	Optical Density

PTPN22	Protein Tyrosine Phosphatase Receptor 22
R	Arginine
Rpm	Revolution Per Minute
RNS	Reactive Nitrite Species
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
SNP	Single Nucleotide polymorphism
TNF-α	Tumor Nacrosis Factor-Alpha
TG	Tri Glyceraldehydes
TBA	Thiobarbutyric Acid
TCA	Tri Carboxylic Acid
TDW	Triple Distilled Water
TIMP	Tissue Inhibitory Matrix Metalloproteinase
TC	Total Cholesterol
U/L	Unit/Litre
VLDL	Very Low Density Cholesterol
VAS	Visual Analog Scale
V	Volume
W	Tryptophan
Wt	Weight
Zn	Zinc

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Chapter 1:

INTRODUCTION

INTRODUCTION

Diabetes is an enigma, genetically inherited, very essentially a metabolic disease that is revealing its secrets slowly to the researchers all over the world. There has been a quick upswing in the number of diabetic patients and this stupendous growth is noted in urban as well as rural areas. Ever increasing industrialization, narrowing urban-rural divide, amplified economic growth, changing dietary norms, lesser or no physical activity and alleviated stress levels among all strata of society are the risk factors behind the devil called Diabetes.

Communicable and nutrition related diseases have mostly overshadowed non communicable diseases (NCDs) including diabetes in low and middle income countries. However, recent times have witnessed a sustained rise of NCDs. The WHO report of 2005 (World Health Report 2005) stated that the contribution of NCDs towards total mortality in India is 52% and this is anticipated to rise to 69% by 2030 (Roglic and Unwin et al., 2005). Thus countries like India are facing the "double burden" of communicable as well as non-communicable diseases. In India, type 2 diabetes is scaling the heights of becoming a potential epidemic with more than 62 million individuals currently diagnosed with it (Joshi and Parikh, 2007; Kumar et al., 2013) and according to 5th edition of International Diabetes Federation (IDF) Atlas of 2011, it shall reach the 100 million mark by 2030 (International Diabetes Federation Atlas, 5th Edition).

The prevalence of diabetes is predicted to double globally from 171 million in 2000 to 366 million in 2030 with a maximum increase in India (Wildet al., 2004). It is predicted that by 2030, Diabetes mellitus may afflict up to 79.4 million people in India while China (42.3 million) and United States (30.3 million) will also face a significant upsurge in the number of affected individuals (Wild et al., 2004; Whiting et al., 2011).

From being considered as the disease of the elderly, diabetes has now become the major cause of morbidity and mortality affecting youth and middle aged as well. Increased prevalence is responsible for putting socio-economic pressure on the most productive age group and health systems in the country (Mohan et al., 2007). 80% of the total burden of diabetes mellitus is from developing countries of which major contributors are India and China.Indians have a high ethnic and genetic susceptibility for the disease, and also have lower threshold limits for the environmental risk factors. It is a matter of major concern that Indians develop T2DM at a younger age than the western populations (Ramachandranet al., 2010).

Table 1.1- Countries with the highest numbers of estimated and predicted cases of diabetes for the year 2000 and 2030 respectively (Adapted from Wild et al., 2004).

	Year 2000		Year 2030	
Rank	country	Diabetic population(in millions)	Country	Diabetic population(in millions)
1	India	31.7	India	79.4
2	China	20.8	China	42.3
3	U.S.	17.7	U.S.	30.3
4	Indonesia	8.4	Indonesia	21.3
5	Japan	6.8	Pakistan	13.9
6	Pakistan	5.2	Brazil	11.3
7	Russian federation	4.6	Bangladesh	11.1
8	Brazil	4.6	Japan	8.9
9	Italy	4.3	Philippines	7.8
10	Bangladesh	3.2	Egypt	6.7

Type 2 diabetes is characterized by hyperglycaemia which in turn results from insulin resistance. Insulin resistance hampers the insulin mediated glucose uptake by muscles and fat causing the glucose concentrations in blood to shoot up. This eventually leads to enhanced uptake by insulin independent tissues. The elevated glucose levels promote oxidant generation with the simultaneous impairment in antioxidant defence by the interplay of various non-enzymatic, enzymatic as well as mitochondrial pathways (King and Loeken, 2004; Mehta et al., 2006). This hyperglycaemia inflicted oxidative stress has been shown to be a contributor towards development of diabetes and its ensuing complications (Reush, 2003).The complications may be macrovascular (coronary heart disease, peripheral heart disease and stroke) or microvascular (neuropathy, nephropathy and retinopathy) (Wu et al., 2014).

Metabolic reactions in body give rise to reactive substances called as free radicals. High concentrations of these may have damaging effects on cellular proteins, nucleic acids, membrane lipids and may even prove fatal. These include ROS (reactive oxygen species) and RNS (reactive nitrogen species) (Tesfamariam, 1994). Activation of ground state oxygen to a ROS requires energy transfer to form singlet oxygen or the superoxide anion via electron transfer. Minute amounts of oxygen are reduced to superoxide ions in mitochondrial electron transport chain during oxidative phosphorylation (Boveris, 1984; Chance et al., 1979). Normally, superoxide anions are speedily converted into H_2O_2 by the chief mitochondrial enzyme, manganese superoxide dismutase (Mn-SOD) in mitochondria and by copper zinc (CuZn-SOD) in the cytosol (Faraci and Didion, 2004; Mendez et al., 2005). H_2O_2 formed is subsequently converted to harmless H_2O and O_2 by the enzyme glutathione peroxidise along with glutathione reductase in mitochondria. The H_2O_2 diffused into the cytosol is

rendered harmless by enzyme catalase in peroxisomes. H_2O_2 can also be transformed to highly deleterious hydroxyl radical in the presence of transition metals like Cu or Fe (Fenton reaction). Further progeny of H_2O_2 derived ROS include hypohlorite, peroxyl and alkoxy radicals. Polyunsaturated fatty acids (PUFA) upon peroxidation also give rise to ROS such as lipid hydroperoxides, conjugated dienes and malonyldialdehyde (MDA) (Taniyama and Griendling, 2003). Genaration of a cascade of ROS from a single ROS follows the free radical chain mechanism (Guziket al., 2002).

To maintain homeostasis in the body antioxidants should be able to scavenge the ROS produced in the body effectively. As ROS are highly reactive with their rate constants in the range of 10^4 -10^9 $M^{-1}S^{-1}$, antioxidants must be equally efficient and act synergistically. When an antioxidant reacts with a ROS, another one should be able to regenerate the previous one. Effective protection against ROS production and action requires coherent antioxidant capacity in aqueous as well as lipid environments of a cell. These antioxidants possess high potential and redundancy in their availability to safeguard cellular build-up and the vital bio molecules. Recent evidences have also shown that antioxidants exhibit altruistic behaviour in their fight against ROS (Masellaet al., 2005; Valkoet al., 2007). For evaluating the status as well as potential strength of oxidative stress present in the body, the primary marker of choice is plasma. Plasma incorporates compounds that combat oxidative stress thus protecting cell and its bio-molecules from damage. The consolidated effort of all the antioxidants present in plasma reflects its total antioxidant capacity (Tiwari et al., 2013). Reduced antioxidant capacity can be linked to increase in diabetic complications like damage to nerves, kidney failure, blindness and cardiovascular events (Styskalet al., 2012).

Antioxidants are broadly classified into two categories namely; enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidise (GPx),

glutathione reductase (GR), catalase belong to the enzymatic group while vitamins A, C and E, glutathione, flavonoids and trace metals like copper, zinc, magnesium and selenium are grouped under non-enzymatic antioxidants (Esteghamatet al., 2008). Glutathione (GSH) is also a chief cellular antioxidant and is essentially the redox buffer of the cell (Masellaet al., 2005; Valkoet al., 2007). Micronutrients are vital for the development of the body and are required on a day to day basis in minuscule amounts. Calcium, magnesium, iron, sodium, phosphorous, chloride and potassium are called macro elements as they have multiple functions in the body. Coupled with vitamins, these commence synthesis of hormones and energise the metabolic pathways. Trace metals on the other hand include boron, copper, cobalt, sulphur, chromium, zinc, selenium, iodine, manganese and fluoride. These are an integral part of cellular and tissue level function. They associate with macro elements and vitamins to augment their effects. They are considered essential for body and have various metabolic traits and functions (Matsumuraet al., 2000).Another interesting molecule is uric acid (UA), which can not only act as a pro-oxidant, hence a marker of oxidative stress but may also behave as an antioxidant (Patterson et al., 2003). In T2DM, hyperuricemia is seen to be associated with early onset of nephropathy (Bo et al., 2001) and in patients with metabolic syndrome it shows elevated risk of myocardial infarction and sudden cardiac death (Brodovet al., 2010).

Life style related risk factors also play a pivotal role in T2DM development. This is clearly evident from the rising incidence of several secondary complications in diabetic subjects. The modifiable risk factors include dietary choices, weight issues, smoking, alcohol intake, lesser physical activity etc. It has been shown by studies that if these factors are tightly controlled, the risk of developing complications can be reduced considerably. Thus, non-pharmacological management can lessen the dependence on

pharmacological interventions (Chaitrikaet al., 2016). Other factors like age, gender, ethnicity, family history and genetic factors constitute the non-modifiable or irreversible risk factors as they are based on inherent genetic/developmental factors which cannot be altered by lifestyle changes (Steyn et al., 2004). The interaction of the above mentioned factors also plays a role in the pathogenesis of metabolic syndrome (MS). Its main components are obesity, glucose intolerance, elevated blood pressure and dyslipidemia. It is attracting the attention of researchers worldwide as it is a key modifiable determinant of T2DM and cardiovascular disease (CVD) (Cameron et al., 2004; Davey et al., 1998; Pekkanenet al., 1995). Prevalence of MS is found to be high among urban Indian population (Misraet al., 2007) and further studies among different sets of populations is needed to gain in-depth knowledge about the syndrome.

Genetic studies also constitute an important part in understanding the patho-physiology of T2DM and its complications. Single Nucleotide Polymorphisms (SNPs), which are single base differences existing between the DNA of different individuals are the most widely present and stable variety of genetic variation in our genomes. As compared to the other conventional markers such as Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP) and Simple sequence repeats (SSR) these are the markers of choice for the risk associated allele detection in human diseases (Eberleet al., 2007). T2DM is a polygenic disorder with many different genes residing on separate chromosomes adding to its vulnerability. A wide range of environmental factors correlate with these genes to give rise to this disorder. Thus, identification of allelic variants among distinct ethnic groups plays a crucial role in T2DM and constitutes one of the most important areas of research in diabetes as it will eventually lead towards better understanding of the disease related complications, their proper treatment, cure as well as prevention.Point mutations in MTHFR (methylene

tetrahydrofolatereductase) gene have been associated with predisposition to developing diabetic retinopathy (Neugebaueret al., 1997; Parvanovaet al., 2002) and diabetic nephropathy (Hultberget al., 1991; Neugebaueret al., 1998; Noiriet al., 2000). Similarly, long standing inflammation and imbalanced immune system plays a crucial role in the development of T2DM. Pro-inflammatory as well as anti inflammatory cytokines are differentially expressed during insulin resistance and stages of T2DM (Navarro-Gonzalez and Mora-Fernandez,2008. Transforming Growth Factor Beta 1 (TGFß1) is one such inflammatory cytokine that prevents macrophage activation. Clinical studies have shown that it is intricately involved in glomerulonecrosis and interstitial fibrosis leading to end stage renal disease (ESRD) (Qian et al., 2008). Ethnic variations in the polymorphism studies have often yielded conflicting results as far as the roles of these mutations are concerned, indicating the necessity for prudent analysis of the research conclusions demanding further follow up studies in T2DM subjects from different ethnic populations.

Role of protein tyrosine phosphatases has been identified in type 1 diabetes. Further investigations based on genetics and immunology studies have highlighted the role of these in type 1 diabetes development and progression. Alterations in signalling pathways within various cell types, causing central as well as peripheral tolerance failures, advancement of pro inflammatory T cell responses and loss of glucose homeostasis regulation have been unravelled by the study of these phosphatases (Cerosaletti and Buckner, 2013). These findings challenge and motivate us to look for the role of these in type 2 diabetes also.

Diagnosis and the treatment aspect of diabetes have been extensively studied but the identification of early biomarkers/novel pathways suggestive of metabolic aberrations related to T2DM development is still unclear. The quickly growing field of

metabolomics has added new dimensions into the pathology of T2DM as well as ways to predict its onset. Metabolomics represents the whole assembly of small metabolites residing in any organism (Oliver et al 1998; Tweeddale et al 1998). Its advantage over genomics or proteomic studies lies in its sensitivity and its potential for analysis of comparatively lesser number of metabolites than corresponding genes or mRNA molecules. Another advantage takes note of the fact that the metabolites studied are the ultimate downstream products of the interplay between genes and environmental factors. Hence, their levels truly demonstrate the activity of metabolic pathways. It thus enables the recognition of short term plus long term physiological changes in the body and typifies a valuable tool for biomarker identification (Friedrich, 2012). Nuclear magnetic Resonance (NMR) constitutes one of the main high throughput metabolomics tool and provides harmonious snapshots representing the metabolome of body fluids such as cerebrospinal fluid, plasma or urine (Bictashet al., 2010).

The ever rising diabetic population in a country like India puts forward many challenges that have to be seriously faced for appropriate diabetes care and management. A diabetic patient faces multitude of problems ranging from lack of proper infrastructure and health care providers, insufficient knowledge update about the disease among general physicians, economic discrepancies in the health care system to the socio-economic onus of the disease on the patient (Viswanathan and Rao, 2013). Furthermore, poor therapy adherence is also an issue among the patients, which is affected by age, education, complexity of treatment regimen, polytherapy, patient physician interaction and various other social and psychological factors (therapy adherence). Proper knowledge and awareness about diabetes, self –care motivation hampers the onset of complications and may reduce the economic burden of the disease. Life style interventions such as preventing obesity, enhanced physical activity and abatement of

smoking are especially beneficial in preventing T2DM (Leung and Lam, 2000). Patient satisfaction must be ensured using multifactorial strategies (therapy adherence). The significance of advocating healthy lifestyles and risk curtailment has been identified as the most prudential way of curbing the menace of diabetes in resource stricken settings. Presently there is no study on type 2 diabetes from north Indian region which has addressed the disease from biochemical, genetics as well as metabolomics point of view. Therefore, the present study was undertaken to explore type 2 Diabetes mellitus with the following objectives.

Objectives

- Screening of Type 2 diabetic patients and controls based upon inclusion and exclusion criteria.
- Analysis of various biochemical, oxidative and antioxidant parameters including fasting plasma glucose level, glycated haemoglobin, MDA, Catalase, SOD, GPx, GR, G6PD and Aldose reductase in normal as well as diabetic subjects so as to determine their status as well as correlation in the given population.
- Estimating genetic polymorphism of TGFß, MTHFR and PTPN22 genes related to diabetes
- To get further insights into disease pathogenesis, proteomic studies are proposed which would help us in better and efficient diagnosis and prognosis of diabetes.

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Chapter 2:

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Epidemiology Of Diabetes In India

History of epidemiology in India is pretty exhaustive. The first ever archived study on diabetes prevalence was conducted in 1938 in Kolkata (then Calcutta). 1% out of 96,300 records examined was found to have diabetes as diagnosed by glycosuria (Chakravarthy, 1938). 1959 saw the detection of diabetes in vast number of individuals in Mumbai (then Bombay) (Patel and Dhirawani, 1959). Similar detection studies were carried out in different parts of the country in 1966 (Berry et al., 1966; Patel and Talwalker, 1966; Rao et al., 1966; Vishwanathanet al., 1966). However, the sampling done at these programs was not systematic and total population of a particular area was not studied.

Pioneer multi-centric studies on diabetes were conducted by Indian council of Medical Research (ICMR) during 1972 to 1975. Urban areas showed a prevalence of 3.0% while 1.3% was the norm in rural areas (Ahuja, 1979). Studies conducted in the 80's exhibited higher prevalence rates in urban areas for ex., 3.1% in Delhi and 5% in Kudermukh, Karnataka (Ramachandran et al., 1988; Varma et al., 1986). The advent of 90's witnessed swift rise in diabetes prevalence across India. The urban area in Chennai which had shown a prevalence of 8.2% now showed a much higher value of 11.6% as reported by Ramachandran et al (Ramachandran et al., 1992; 1997). Non Communicable Diseases (NCD) risk factor surveillance was taken up by ICMR and World Health Organization (WHO) in 2005 across five states in India to monitor uninterrupted scrutiny of NCD risk factors. 40,000 people in the age group of 15-64 years representing rural, semi urban and urban areas equally, participated in the study. The overall prevalence reported in the study was 4.5% with urban areas being the

frontrunners showing a prevalence of 7.3% followed by 3.2% and 3.1% prevalence in semi urban and rural areas.

Thus, there have been significant changes in the epidemiology of diabetes in India over the years. Since the occurrence of non-communicable diseases and lifestyle disorders is on an upsurge, there is an urgent need to study the changes and estimate the prevalence of diabetes from different parts of the country. This would contribute in proffering latest data for type 2 diabetes in India.

Some of the major studies on prevalence of diabetes carried out in the country are listed in the table 2.1 (Devi et al., 2016) –

Table 2.1: Studies on prevalence of diabetes in India since 1990s

Authors	Year of publication	Place	Prevalence (%)
Ramachandran A et al.	1992	Chennai	8.2
Ramachandran A et al.	1997	Chennai	11.6
Shah SK et al.	1999	Guwahati	8.2
Raman KV et al.	1999	Trivandrum	16.3
Zargar AH et al.	2000	Kashmir Valley	6.3
Iyer SR et al.	2001	Mumbai	7.5
Misra A et al.	2001	Delhi slum	11.2
Mohan V et al.	2003	Chennai	12.0
Gupta R	2004	Jaipur	16.8
Mohan V	2005	Chennai	15.5
Prabhakaran D	2005	Delhi	15.0
Reddy KS	2006	National	10.1
Ravikumar P	2011	Chandigarh	11.1
Mustafa N	2012	Jabalpur	11-18 (range)
Gujral UP	2015	Chennai	38.0

2.2 Classification Of Diabetes

Insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) terms were proposed by WHO (World Health Organization) in 1980 and 1985 respectively. These terms have become obsolete nowadays and the new classification of 1999 recognizes four types of diabetes mellitus (WHO, 1999).

2.2.1 Type 1 Diabetes Mellitus (T1DM)

It is signified by beta cell destruction emanating from an autoimmune system, ultimately heading off to complete insulin deficiency (Kumar and Clark, 2002). It is generally distinguished by existence of anti-glutamic acid decarboxylase, islet cells or insulin antibodies which ascertain the autoimmune pathways that cause beta cells destruction. Finally, all type 1 patients require insulin to regulate glycaemic control (Kumar and Clark, 2002).

2.2.2 Type 2 Diabetes Mellitus (T2DM)

T2DM captures 80-90% of all the diabetes cases. Defective insulin secretion and/or insulin resistance is the major cause of its etiology. Hypertension and dyslipidemia are usually found among T2DM patients. Family history, obesity, sedentary habits and old age are risk factors for T2Dm (Baynest, 2015).

2.2.3 Gestational Diabetes Mellitus (GDM)

It is identified in women who develop diabetes mellitus during pregnancy (usually during the third trimester). Women who develop type 1 diabetes during gestation and those with undiagnosed asymptomatic T2DM revealed during pregnancy, both are grouped under gestational diabetes mellitus (Baynest, 2015).

2.2.4 Other types of Diabetes (monogenic diabetes)

Different types of diabetes of known etiologies are clubbed together under this section. People with genetic abnormalities of beta cell function (formerly recognised as MODY

or maturity onset diabetes in youth) or with defective insulin action; persons having pancreatitis, cystic fibrosis, acromegaly and persons with defective pancreas owing to drugs, infections or chemicals are categorised under it and they constitute less than 10% of diabetes mellitus cases (Baynest, 2015).

2.3 Diagnosis of Diabetes

American Diabetes Association (ADA) recommendations (1997) for diagnosis of diabetes lay emphasis on fasting plasma glucose (FPG), while WHO relies on oral glucose tolerance test (OGTT) (Gillett, 2009)-

2.3.1 Random plasma glucose test- Simplest test with no need of fasting before taking the test. A test value ≥200mg/dl indicates diabetes, which has to be reconfirmed.

2.3.2 Fasting plasma glucose test- At least eight hours of fasting is required before taking this test. Glucose levels ≥126mg/dl on two or more occasions on different days confirms the diagnosis of diabetes.

2.3.3 Oral glucose tolerance test- this test is required when value of random plasma glucose level is between 160-200 mg/dl and that of fasting plasma test is between 110-125 mg/dl. It checks body's response to glucose and requires fasting for at least eight but not greater than sixteen hours. After determination of fasting glucose level, 75 gm of glucose is given and blood is tested every 30 minutes for 2 to 3 hours. If the test values come out to be < 140 mg/dl, the person is non-diabetic, but a value of 200mg/dl or higher confirms a diabetes diagnosis (Gillett, 2009).

2.4 Pathophysiology Of Type 2 Diabetes Mellitus

Impaired insulin secretion as well as insulin resistance together leads to pathophysiology of T2DM. Progressive deterioration of β-cell function is a hallmark of pathophysiology of T2DM (see Figure 2.1a and 2.1b).

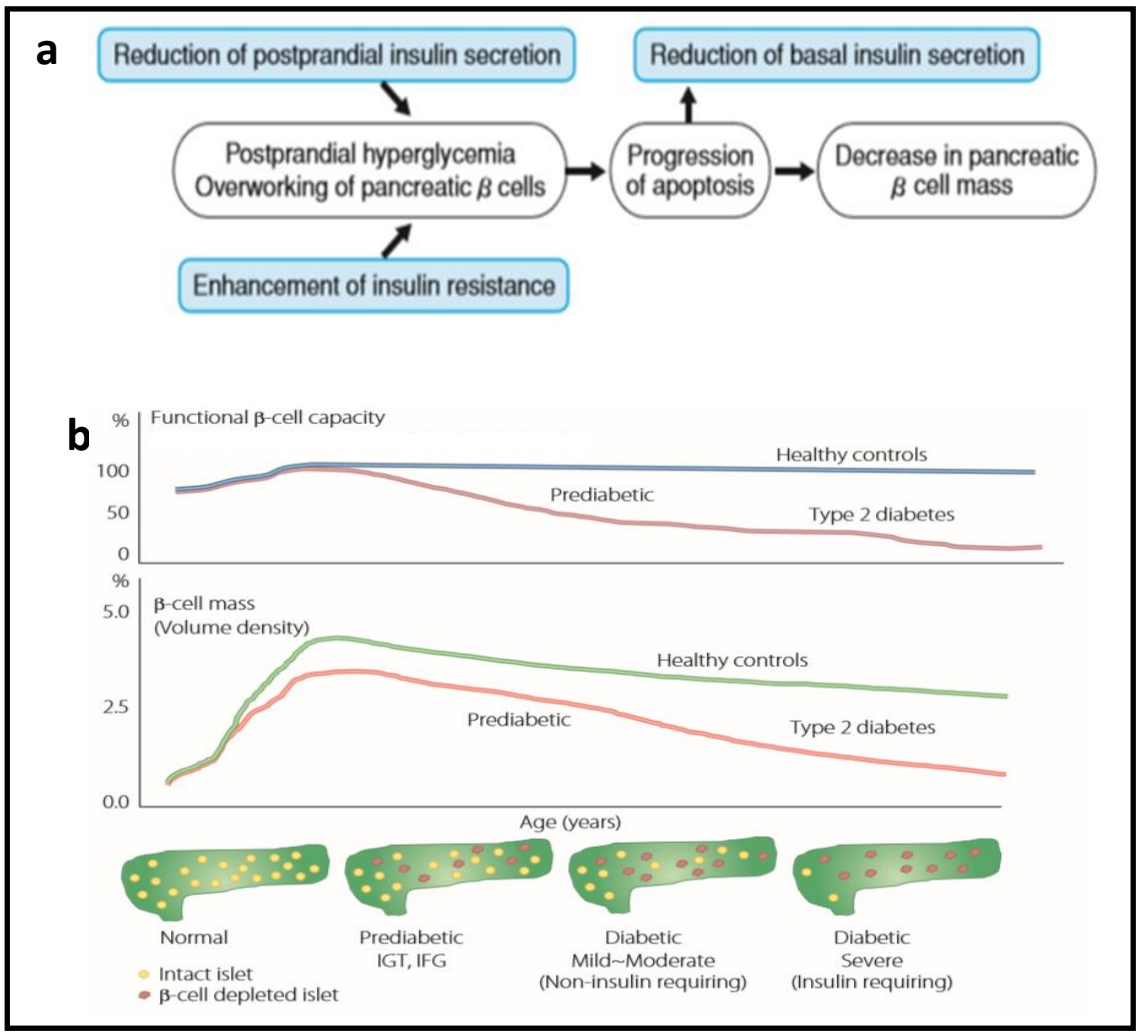


Figure 2.1: Pathophysiological progression of T2DM.
(a) Impaired insulin secretion and insulin resistance and T2DM (adapted from Kaku, 2010), (b) Progressive deterioration of β-cell function during development of T2DM (adapted from Yagihashi, 2012).

2.4.1 Insulin resistance

Himsworth in 1930s first described insulin resistance as the incapability of target tissues to suitably reply to flowing concentrations of insulin which on the other hand is effectual in normal healthy individuals. Usually, insulin attaches to insulin receptors located on target cells, culminating in a succession of cellular affairs that marshal towards intracellular transport of glucose and metabolism. Two main insulin receptive organs, in charge of proper glucose homeostasis are liver and skeletal muscles. Skeletal muscle is responsible for 75% of Insulin mediated glucose uptake, which is either instantly used up or stored in form of glycogen. Liver which is the chief organ responsible for producing, consuming and storing of glucose and lipids, also witnesses the storage of glucose as glycogen and diminished gluconeogenesis thus decreasing hepatic glucose output. Furthermore, within the liver insulin signals a minimized requirement for lipid metabolism causing movement of free fatty acid for deposition in adipose tissue. Disfigured insulin action and impaired insulin sensitivity in liver appreciably adds to the pathogenesis of T2DM (Fritscheet al.,2008).

Prevalence of insulin resistance is on rise worldwide and it is a key feature of majority of T2DM patients and about two-thirds of those with impaired glucose tolerance (Bonora et al., 1988). This significantly increases the chances of both the groups of developing CardioVascular diseases (DECODE Study Group, the European Diabetes Epidemiology Group, 2001;Haffner et al., 1998; National Diabetes Data Group, 1995; Saydah et al., 2001).

2.4.2 Impaired insulin secretion and β-cell dysfunction

β-cells of pancreas, by secreting insulin with increasing concentration of glucose maintain glucose homeostasis in the body. Insulin causes lowering of blood glucose level by stimulating its uptake into adipose tissue and skeletal muscle and by hindering hepatic glucose production. β-cells function deteriorates before hyperglycaemia sets in. Various mechanisms involved in T2DM such as chronic hyperglycemia (Donath andHalban, 2004), endoplasmic reticulum stress (Harding and Ron 2002), chronic hyperlipidemia (Poitout and Robertson 2002), inflammatory cytokines (Donath et al., 2003) and oxidative stress (Kaneto et al., 2006), participate in inciting beta cell apoptosis and lessen the ability of beta cell to counterbalance insulin resistance. When beta cell dysfunction is seen in light of retarded insulin sensitivity, substantial evidences validate the untimely collapse of insulin secretion in the pathogenesis of T2DM (Lin and Sun, 2010).

Thus type 2 diabetes initiates with peripheral insulin resistance and ends with beta cell dysfunction or failure. Peripheral insulin resistance is the diminished reply to insulin in liver, fat tissue and skeletal muscle. It usually occurs way before the onset of hyperglycaemia. Lowering of glucose uptake occurs in skeletal muscles due to insulin resistance. Similar suppression of hepatic glycogenolysis and gluconeogenesis raises the outflux of glucose from liver. Fat tissues witness enhanced lipolysis and elevated flux of free fatty acid (FFA) into circulation owing to resistance to antilipolytic mechanisms of insulin. Long standing elevation in levels of FFA can lead to pancreatic beta cell death due to lipotoxicity. To combat peripheral resistance, beta cells show enhancement in mass, secreting larger amount of insulin, thereby causing hyperinsulinemia. However, a stage comes when beta cells can no longer endure the ordeal and give in, causing plasma glucose levels to rise. Amplified glucose levels may confer damage by causing

gradual loss of islet beta cells and the stage is set for the development of uninterrupted hyperglycaemia.

2.4.3 Hyperglycaemia

Hyperglycaemia selectively damages some cell types and not others. This selective targeting shows the inability of these cells to down regulate their glucose uptake with increase in concentration of extracellular glucose. Those cells which are able to maintain an inverse ratio between glucose influx and its extracellular concentration are not the victim of hyperglycaemic damage. Conversely, cells of vascular endothelium are main targets of hyperglycaemia induced damage as their glucose influx rate remains unaltered even when outside concentration of glucose becomes high causing intracellular hyperglycaemia.

There are 5 mechanisms through which hyperglycaemia take its toll on the body-

(a) Elevated flow of glucose as well as other sugars via polyol pathway

(b) Heightened intracellular generation of Advanced Glycation End products (AGEs)

(c) Amplified expression of AGE receptors and its activating ligands

(d) Energization of isoforms of Protein Kinase C

(e) Over excitation of hexosamine pathway

2.5 Diabetic Co-morbidities

Diabetes is a huge burden to the society and many complications are associated with it. These are divided into two types based on their duration-

2.5.1 Acute metabolic issues- involve hyperglycaemic coma, ketoacidosis and hypoglycaemia (English and Williams, 2004; Umpierrezet al., 2002).

2.5.2 Systemic long standing co-morbidities- these include coronary artery diseases, nephropathy, neuropathy and retinopathy (American Diabetes Association, 1998).

Another classification used to denote the type of morbidity is-

2.5.3 Macro-vascular complications- include coronary heart disease, peripheral vascular disease, stroke

2.5.4 Micro-vascular complications- include nephropathy, neuropathy and retinopathy.

An array of ingredients affects the progression of these co-morbidities. Insulin secretion dwindles with increasing age and this waning off may be hastened by genetic elements. Insulin resistance prepares the way for the outset of T2DM and is generally escorted by other cardiovascular risk factors such as dyslipidemia, prothrombotic factors and hypertension (Mezzettiet al., 2000). Studies have furnished indisputable confirmation on pivotal role of extended hyperglycaemia in the progression of long standing diabetic co-morbidities (Jaganjacet al., 2013; Paneniet al., 2013; Leung and Lam, 2000). Free radicals contribute in the same, owing to their potential of injuring DNA, lipids and proteins. Various studies have linked oxidative stress and diabetes using markers of DNA damage and lipid peroxidation (Ayepolaet al., 2014).

Insulin resistance emanating from sedentary lifestyle and obesity behave as substrate for genetic vulnerability towards the development of these complications (DeFronzo and Ferrannini, 1991). As food influx directly affects the quantity of insulin to be released to counteract the blood glucose levels, food rich in carbohydrate may be instrumental in diabetes pathology. Post-prandial blood glucose levels are most affected by

carbohydrate rich diet which is the paramount element for meal associated insulin demands. Thus, low carbohydrate diet is useful in amending some of the damages conferred by diabetes (Al-Khalifa et al., 2011).

2.5.5 Dyslipidemia: It classically involves elevated levels of plasma triglycerides, LDL cholesterol along with decreased levels of HDL cholesterol. Elevated free fatty acid flux is considered responsible for these variations in the lipid profile (Mooradian, 2009). It is one of the major risk factors for cardiovascular diseases resulting from diabetes and is also regarded as a comorbidity affecting the development of kidney complications in diabetes (Ansquer et al., 2005). It is also influential in the progression of diabetic neuropathy (Davis et al., 2008). Some studies have shown that continuously raised plasma triglycerides levels corroborated with faster development of diabetic neuropathy (Rajamani et al., 2009). Dyslipidemia is thus highly relevant for development of diabetic comorbidities.

2.6 Risk factors for T2Dm

The major risk factors for development of T2DM are classified into **Irreversible risk factors** (based on genetic/developmental elements that cannot be modified by lifestyle changes, ex., Ethnicity, family history, age and gender) and **Reversible risk factors** (based on discrepancies in lifestyle that can be addressed by modifications in the same, for example, obesity, physical inactivity, dietary control, smoking, alcohol intake).

2.6.1 Ethnicity

Prevalence of T2DM fluctuates appreciably among different ethnic populations residing in outwardly alike habitat (King and Rewers, 1993). Asian Indians show higher prevalence rates for diabetes when compared

with native populations in U.K., South Africa, Fiji, Africa and in Caribbean (Mather et al., 1998; Omar et al., 1994; Zimmet et al., 1983).

2.6.2 Family History-

The actual risk of having T2DM is enhanced by 2-6 fold if either of the parents or sibling already has the disease (Everhart et al., 1985). It is thus a rough yet practical way of evaluating a person's inherited susceptibility to T2DM. Various studies in India and abroad have reported the presence of first degree family history in 75% of the T2DM patients, indicating a strong genetic connection in Indian diabetic subjects. Insulin resistance is reported to be a distinguished feature of asian Indians (Ramachandran, 2007). Comparative study done among Asian Indians, Europeans and other ethnic groups has revealed that Asian Indians have enhanced insulin response than other populations, both in fasting as well as in response to glucose. Insulin resistance and the resulting hyperinsulinemia as a counterbalancing act leads to chronically enhanced insulin and glucose values in blood (hyperglycaemia) which is the ultimate diabetes precursor (Chowdhary andLasker, 2002).

2.6.3 Age

T2DM incidence is usually low before the age of 30 years in most populations but progresses rapidly with advancing age. The prevalence and incidence rates between the two genders seem to vary from one population to another, but the differences are relatively small. In affluent societies, T2DM occurs in middle to older age groups but in developing countries like ours because of the younger age distribution of the population, vast numbers of cases happen in young and middle aged adults. Asian Indians witness diabetes onset atleast 10-15 years earlier than Caucasians (The DECODE-DECODA

Study Group, 2003; Ramachandran et al., 2004). National urban diabetes survey (NUDS) in India has reported that incidence of diabetes occurs at less than 50 years of age (Ramachandran et al., 2001). Indians have many folds greater diabetes prevalence compared with their European counterparts for all age groups as documented by International Diabetes Epidemiology group (The DECODE-DECODA Study Group, 2003). Recent studies in India have also shown the trend of decrease in diabetes onset (Ramachandran et al., 2008). Number of patients under 40 years of age is on rise since the last decade (Ramachandran et al., 2008).

2.6.4 Obesity

Prevalence of T2DM increases with increase in obesity (Mokdad et al., 2001). Central adiposity includes vast quantities of abdominal fat which in turn is made up of visceral and subcutaneous fat. Visceral fat enhances diabetes risk as well as hyperlipidemia by promoting insulin resistance. In non-obese asian population, the pattern of body fat signified by greater upper body adiposity, measured in form of waist to hip ratio was reported to be a more alarming risk factor for T2DM than general obesity (Gupta et al., 2007; Ramachandran et al., 2002). Ceaseless relationship is observed between all obesity markers (BMI, Waist to Hip ratio) with coronary risk factors like hypertension and metabolic syndrome (Gupta et al., 2007). Abdomial obesity is a vital ingredient of metabolic syndrome. Susceptibility of different races towards insulin resistance and metabolic syndrome has been shown in studies and Asian Indians have enhanced vulnerability for both (Abate et al., 2001; McKeigue et al., 1991; Snehalathaand Ramachandran, 1999). Occurrence of high insulin response even at lower BMI can be understood by adiposity of upper body in Asian Indians. Studies comparing topography of body fat between migrant Asians and Caucasians have validated these findings (Chandaliya et al., 1999; Raji et al., 2001). For a given BMI, Asian Indians have greater

fat percentage compared with Caucasians (Raji et al., 2001). With increasing BMI, the risk of developing T2DM increases in a "dose dependent" fashion (Mokdad et al., 2001). Even a moderate weight loss can upscale insulin action, reduces hyperglycaemia and lessen dependence on medication (Torgerson et al., 2004).

2.6.5 Physical activity

Physical activity is a useful partner of dieting during any weight management regime. It aids in weight loss as well as prevents its regain (Wing, 2002). Increasing the quantity as well as quality of physical activity greatly reduces the risk of cardiovascular diseases. Reduced physical activity elevates diabetes risk by upto 14% while brisk walking for at least an hour per day decreases the risk by 34% (Hu et al., 2003).

2.6.6 Dietary control

WHO has acknowledged the significance of diet regulation in diabetes and has given its approval regarding nutrient distribution in diabetes (WHO, 2006). Main points of dietary guidelines issued by the American Diabetes Association and American Heart Association are as follows (Franz et al., 2003; Krauss et al., 2000)-

(a) consumption of different kinds of vegetables, grains, fruits, low/non-fat dairy products, legumes, poultry and fish.

(b) limited intake of foods rich in transfatty acids, saturated fat, cholesterol

(c) salt limit should be 6 gm/day (2400 mg sodium) by opting for low salt foods

(d) alcohol intake should be limited to 2 drinks per day for men and 1 for women per day.

2.6.7 Smoking

Smoking is also connected with elevated diabetes risk. Willey et al., in their study reported that frequent smokers had more chances of developing diabetes (relative

risk=1.61) than occasional smokers (risk =1.290. ex-smokers had further reduced risk (1.23) as compared to active smokers.

2.6.8 Alcohol Intake

A study has reported that the relationship between alcohol intake and glycaemic control is inversely proportional thus concluding that diabetic co morbidities can be curtailed by reducing alcohol consumption (Ahmed et al., 2008).

2.6.9 Stress

The effect of stress whether physical or mental is strongly seen in the individuals during the course of diabetes, especially in those with strong genetic susceptibility (ref 65 66 of india new). Stress, sedentary nature of work, unhealthy food habits plague the economically well off youth of today (Maudgalya et al., 2006).

2.7 Oxidative Stress and T2DM Complications

2.7.1 Overview of oxidative stress:

Oxygen is the lifeline of aerobic world. Atmospheric build-up of oxygen is responsible for the aerobic evolution of organisms on earth. Molecular oxygen first appeared 2.5 billion years ago with advent of blue green algae, the pioneer photosynthetic organisms (Semenza, 2007). But the same oxygen can be deleterious when it gives rise to a progeny of reactive species that in turn promote necrosis and cell death.

Oxidative stress can be defined as perturbation caused when the balance between pro-oxidants and antioxidants goes haywire, usually in favour of the former. In other words, it occurs during surplus generation or/and inadequate expulsion of free radicals for ex., ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) (Johansen et al., 2005). It disfigures the structural as well as the functional harmony of healthy cells

making them prone to many diseases. The damage becomes more devitalizing and mounting if the anti-oxidant concentration is finite (Mark Percival 1996). It functions as an intermediary of insulin resistance and its development to glucose intolerance and ultimately to diabetes mellitus, promotes the surfacing of atherosclerotic morbidities. Cardio-myopathy and atherosclerosis occur as a result of pathway selective insulin resistance in T2DM patients which enhances the generation of ROS in mitochondria from free fatty acid and inactivates the protective anti atherosclerosis enzyme. Insulin sensitive tissues like heart, liver and muscle get subjected to high concentrations of free fatty acids and suffer oxidative damage. Non-insulin sensitive tissues like kidney, nervous system and eye too face hyperglycaemia as well as elevated levels of free fatty acids and become slave to ROS induced diabetic co-morbidities. During the course of diabetic complications, the ramifications of oxidative stress may not elicit mass destruction of tissues, nevertheless damage incurred upon individual cells may be potent enough to initiate breaks in DNA strands and inflict cell death (Du et al., 2003). Hyperglycaemia incited oxidative stress may also alter normal gene expression. It may promote expression of protective genes which help cells against oxidant driven damage of DNA, lipids and proteins. Foe ex., genes of free radical scavenging enzymes and DNA repair enzymes (Hancock et al., 2001, Liu et al., 2001, Napoli et al., 2001). If homeostasis is not restored successfully or completely then oxidative stress may induce cell death by activating signal transduction pathways, proapoptotic genes and repression of antiapoptotic genes.

Oxidative stress in diabetes thus interferes with normal cellular physiology and participates in the onset of micro as well as macro vascular complications (Paneni et al., 2013). It is of utmost importance for the islet as it faces minimal amount of intrinsic

antioxidant defense. Oxidative stress may occur due to various factors such as aging, toxicity, action of drugs, inflammation and/or addiction (sies, 1985).

2.7.2 Overview of free radicals

Free radicals are reactive species having one or more unpaired electron that have short life span. Their damaging behaviour involves passing the unpaired electron causing oxidation of molecules and cellular components. They are highly reactive and unstable at the same time (Bansal and Bilaspuri, 2011). Exogenous as well as endogenous substances contribute in production of free radicals inside cells and their vicinity (see Table 2.2).

Table 2.2: Sources of free radicals (adapted from Ajuwon et al., 2015)

Endogenous sources	Exogenous sources
Mitochondrial electron transport chain	Radiation (UV light, X-ray and γ-radiation)
Neutrophils and macrophages during inflammation	Environmental pollutants and toxins
Xanthine oxidoreductase, NADPH oxidase	Cigarette smoke, excessive alcohol, high-calorie diet
Microsomal oxidation in endoplasmic reticulum	Heavy metals
Myeloperoxidase (Phagocytes)	Infectious agent
Lipoxygenase, cyclooxygenases, prostaglandin synthase	Strenuous exercise

They can result from non-enzymatic reactions of organic compounds having oxygen plus ionizing radiations (Pham-Huyet al., 2008). They are basically of three types-

(a) ROS- Reactive Oxygen Species (Bansal and Bilaspuri, 2011)

(b) RNS- Reactive Nitrogen Species (Droge and Wulf, 2011)

(c) RCS- Reactive Chlorine Species (Freidovich, 1999)

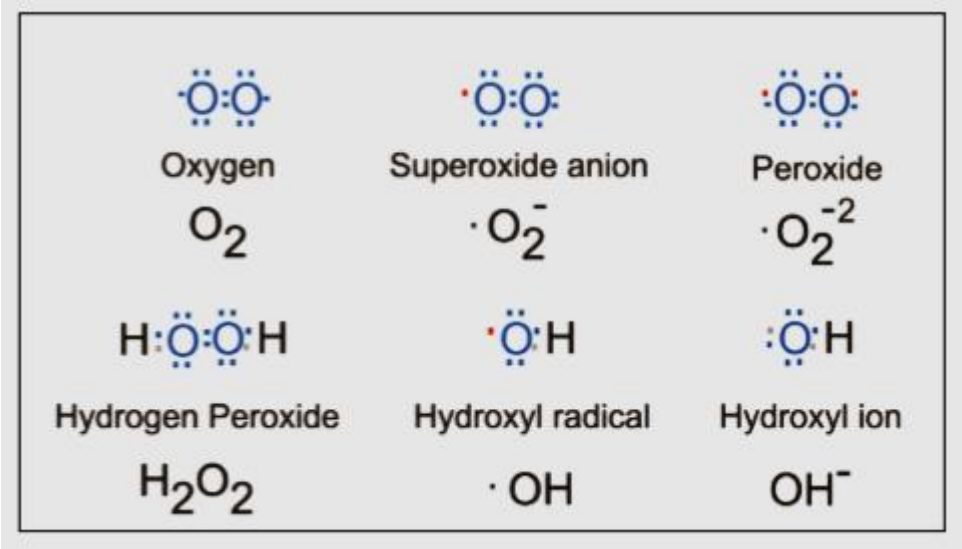


Figure 2.2: Some common reactive oxygen species (adapted from Held, 2014).

It has been reported that excessive generation of reactive oxygen species (ROS) by mitochondria is responsible for free radical mediated cellular damage (Du et al., 200; Nishikawa et al., 2000). Superoxide is the primary oxygen free radical produced by mitochondria which then gives rise to other reactive species that cause havoc to cells in various ways (Wallace, 1992).

2.7.3 Biological role played by free radicals

Some amount of ROS is vital for proper metabolic functioning as they are crucial for excitation of various signalling pathways within the cell, for ex., MAPK (Mitogen Activated Protein Kinase) and ERK (Extracellular signal Regulated Kinase) pathways that transform gene expression and initiate cell death in coordination with superoxide

dismutase (Cho and Wolkenhauer, 2003). Reactive nitrogen species generated by neurons behave as neurotransmitters and those produced by macrophages function as immunity moderators. They are also accountable for angiogenesis, leucocyte adhesion, vascular tone and thrombosis. Moreover, signal transduction, gene transcription and regulation of other cellular activities also witness the involvement of ROS (Fang et al., 2002). Neutrophils and macrophages generate ROS to exclude antigens during respiratory burst (Freitas et al., 2010).

2.7.4 Scavenging of free radicals

To neutralize the damaging effects of these free radicals, various homeostatic mechanisms are put forward by the body. As displayed in Figure 2.3, these include production of antioxidants either exogenously or endogenously which will help in protection of cells against deleterious effects of free radicals and would eventually aid in prevention of diseases.

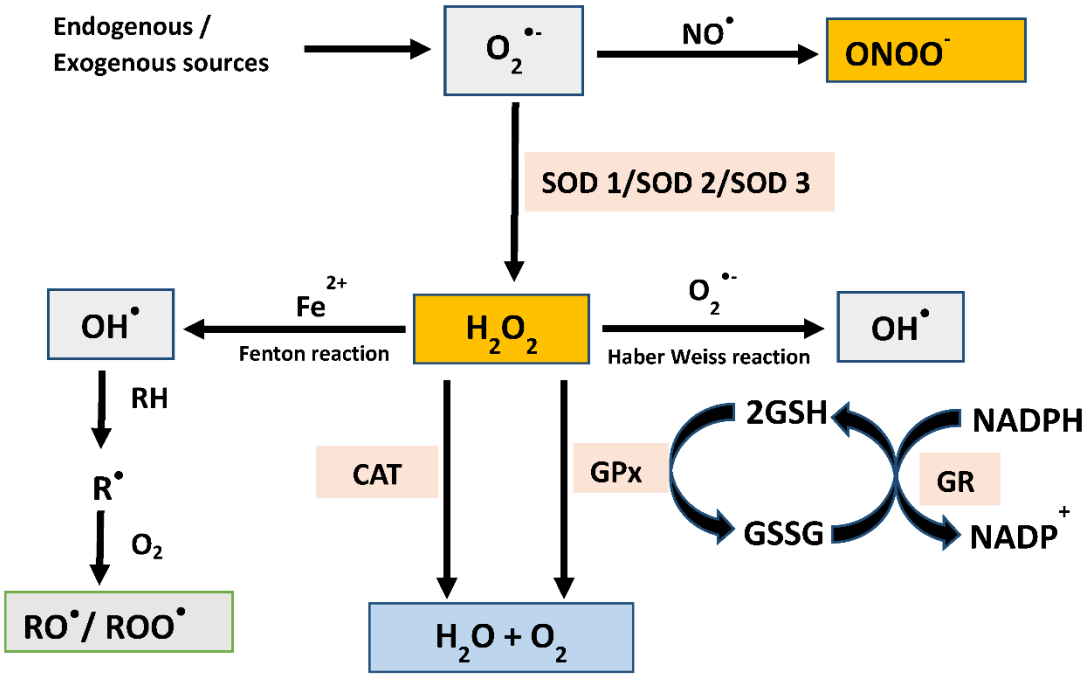


Figure 2.3: Generation and scavenging of free radicals.
abbreviations- CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; RH, lipid membrane; R., alkyl radical (adapted from Ajuwon et al., 2015).

2.7.5 Markers of Oxidative Stress

2.7.5.1 Lipid peroxidation: Lipids are found to be one of the major victims of ROS. Hydroperoxides cause noxious impact on cells directly as well as through their dissipation into highly injurious hydroxyl radicals. Their reaction with transition metals like copper and iron gives rise to stable aldehydes like malondialdehyde (MDA), which is detrimental to cell membranes (Halliwell and Chirico, 1993). Per-oxyl radicals detach hydrogen molecule from lipids generating hydroperoxides that further proliferate the free radical chain (Lobo et al., 2010). MDA is considered a principal biomarker for free radical moderated lipid deterioration plus oxidative stress (Shodehinde andOboh, 2013). Elevated MDA levels in diabetics hint at the involvement of peroxidativeimpairmrnt in the progression of diabetic comorbidities. The same is also indicative of dwindling defence system of enzymatic as well as non-enzymatic antioxidants (Saddala et al., 2013). Oxidised lipids generate MDA as degradation product and the process involves emergence of prostaglandins from polyunsaturated fatty acid (PUFA) (Pandey and Rizvi, 2011). Enhanced MDA values in serum, plasma and various other tissues have been documented in diabetic subjects (Bandeira et al., 2012; Moussa, 2008). Raised lipid peroxidation advocates a direct bonding between high glycemic values and oxidative stress in diabetes (Bandeira et al., 2012; Levine et al., 1990). In diabetics, it has been shown to induce various secondary distortions including neural disorders and atherosclerosis (Baynes, 1991; Ramesh et al., 2012). Increased MDA values observed in serum of hyperglycaemic mice led researchers to propose that enhancement in lipid peroxidation aggaravated the manifestation of myocardial infarction via activation of

NADPH oxidase (Yang et al., 2009). RBCs as well as serum, exhibiting significantly elevated concentrations of Thiobarbituric acid reactive substances (TBARS) and diminished activities of erythrocytic antioxidant enzymes have been outlined in diabetes (Singh and Shin, 2009; Varashree and Bhat, 2011).

2.7.5.2 Superoxide Dismutase (SOD): The antioxidant enzyme responsible for dismutation of harmful superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen is superoxide dismutase. (Faraci and Didion, 2004; Wang et al., 2012). It is the first line of defense and gallantly protects the cell against the detrimental effects of ROS. Mammals havethree distinct forms of this enzyme: Cu-Zn-SOD, extracellular SOD amdMn-SOD. Each of them originates from a discrete gene (Beyer et al., 1991; Xia, andF¨orstermann, 2012)-

(a) Cu-Zn-SOD or SOD1 (EC 1.15.1.1)- resides in cytosol

(b) Mn-SOD or SOD2 (EC 1.15.1.1)- found in mitochondria

(c) EC-SOD or SOD3 (EC 1.15.1.1)- localized in extracellular matrix of tissues like skeletal muscle, pancreas and blood vessels. It is the chief forager of super oxide radicals (Fattman et al., 2003; Oury et al., 1996; Zelko et al., 2002). Elevated values of EC SOD contribute towards an overall 6 times raise in the total SOD activity of the islets; hence superoxide radicals released into the extracellular space have no role play in beta cell destruction (Sandstr¨om et al., 2002).

Enhanced SOD levels corroborate with diminished oxidative stress, reduced exit of cytochrome c from mitochondria and decreased neuronic apoptosis. In mice, it is shown to prevent diabetes inflicted damage thus hinting at the major contribution of SOD in apoptosis regulation (Kowluruet al., 2006). No distinguishable activity pattern of SOD is seen as far as gender/species of animal, duration of diabetes or tissue studied is considered. Similarly, activity may be depressed or enhanced in RBCs, diminished in

plasma and retina and elevated in pancreas. Antioxidant supplementation along with SOD mimetics or SOD overexpression aimed at reducing oxidative stress has shown to be effective in preventing diabetes (Wang et al., 2011).

2.7.5.3 Glutathione level: Glutathione is a tripeptide (gamma-l-glutamyl-L-cysteinylglycine) omnipresent in mammalian tissues, which protects them from oxidative stress (Lu, 2013). It is regarded as abiomarker of redox disparity in the cell (Rizvi and Chakravarty, 2011). The enzymes responsible for maintaining glutathione homeostasis in the cell are glutathione peroxidise (GPx) and glutathione reductase (GR). Using GSH as a reducing agent, GPxcatalyzes the reduction of H_2O_2 and organic peroxides to water and corresponding alcohol thus inhibiting the formation of free radicals. Glutathione reductase reduces the GSSG to GSH via oxidation of NADPH to NAD+. Thus, together these enzymes convert harmful peroxide to water and revert oxidized glutathione disulfide (GSSG) back into reduced glutathione (GSH) (Maritimet al., 2003) (also see Figure 2.4).

However, this whole mechanism can be over powered by copious generation of ROS (Morris et al., 2013). Depressed glutathione levels may contribute towards oxidative stress in T2DM (Dincet al., 2002). Plasma GSH/GSSG ratio is shown to be significantly decreased in T2DM as compared to controls (Calabrese et al., 2012). Inflammation, Hyperlipidemia and deranged antioxidant status are the usual repercussions of T2DM owing to diminished GSH/GSSG ratio (Das et al., 2012). Their abnormal levels make the cells susceptible to oxidative stress and to further damage. Deranged GSH levels is indicative of beta cell dysfunction and involvement in pathogenesis of long standing diabetic comorbidities (Livingstone and Davis, 2007).

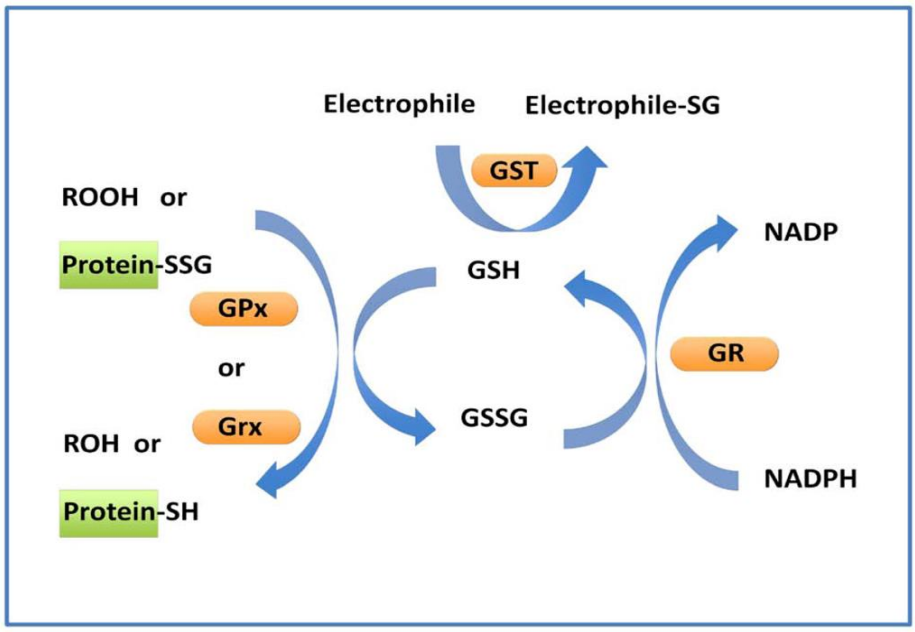


Figure 2.4: Anti-oxidant properties of glutathione.

Cellular function requires a dynamic ratio of GSH:GSSG. The enzymes (high-lighted in orange), glutathione peroxidase (GPx), glutaredoxin (Grx), glutathione reductase (GR), and glutathione-*S*-transferase (GST) use GSH as a substrate, and their actions result in fluctuations in the GSH:GSSG ratio. The scheme above shows the outcome of the reaction and the direction of GSH or GSSG production. Both electrophile-SG adducts and GSSG can be extruded from cells, requiring re-synthesis of GSH; whereas intracellular GSSG is re-cycled to GSH by GR and NADPH.

2.7.5.4 Catalase: It is an antioxidant enzyme found nearly in all living organisms. Its chief role lies in the management of hydrogen peroxide metabolism. Hydrogen peroxide overload is deleterious for the health of DNA, RNA, proteins and lipids (Takemoto et al., 2009). It nullifies the effect of harmful hydrogen peroxide by catalysing its conversion into oxygen and water. Catalase deficiency has been linked to increased diabetes risk. Beta cells of pancreas, upon experiencing catalase deficiency, undergo oxidative stress and ultimately suffer failure. As these cells are mitochondria rich, this organelle might be the culprit behind ROS generation (G´oth and Eaton, 2000). Reduced catalase activity may result in homolyticanemia and methemoglobinemia which can be due to deficiency of G6PD and may injure heme proteins, confer cell

death and along with redox metal ions generate highly detrimental hydroxyl radicals (G´oth and Bigler, 2007; G´oth et al., 2005). Upon exploration of hyperglycemia inflicted functional reforms: hydrogen peroxide, superoxide production, polarization of mitochondrial membrane and gene expression fingerprints of associated enzymes within endothelial cells, it was suggested that hyperglycaemia aided in H_2O_2 production, overpolarization of mitochondrial membrane and downregulation of catalase gene expression (Patel et al., 2013).

2.7.5.5 Aldose reductase: Under normoglycemic conditions majority of the cellular glucose enters glycolytic pathway, with only 3% of non-phosphorylated form entering the polyol pathway (Morrison et al., 1970). However, hyperglycaemia induces elevated glucose flux via polyol pathway (>30% of entire glucose metabolism) (Yabe-Nishimura, 1998). The reduction of glucose to sorbitol, which is the rate limiting step of this pathway, is catalysed by aldose reductase (AR) using NADPH. The NADPH thus consumed, leads to diminished glutathione level, which also requires it for conversion into its stable reduced form, causing oxidative stress (Cheng and Gonzales, 1986). Increased glucose flux through polyol pathway is thus associated with plethora of diabetic complications via many pathways like, activation of PKC, altered cellular redox status and elevated oxidative and glycation stress, disturbances in Na^+/K^+ ATPase activity (Steele et al., 1993). It leads to excessive accumulation of intracellular ROS in various tissues of T2DM including heart, neurons, eyes, kidneys and vasculature. Thus hyperglycaemia through polyol pathway induced oxidative stress may cause atherosclerosis and myocardium leading to severe morbidity and mortality (Heather and Clarke, 2011). Cultured cells amidst high glucose environment display similar AR-mediated increase in ROS generation, validating the role of AR as a crucial ingredient in pathogenesis of various diabetic comorbidities.

2.7.5.6 Glucose-6-Phosphate-Dehydrogenase (G6PD): G6PD is the first as well as rate limiting enzyme of pentose phosphate pathway which generates NADPH from NADP (Kletzien et al., 1994; Wood, 1986). Ratio of NADPH/NADP regulates the activity of G6PD. As the ratio declines, G6PD activity increases to generate more NADPH. Its activity is shown to be directly correlated with cell growth (Stanton, 2012). Elevated G6PD activity has been shown to be vital in preventing ROS actuated cell death (Tian et al., 1999). Antioxidant system of cells relies heavily on generation of NADPH for proper functioning. Its three chief supporters in cells are glutathione, catalase and superoxide dismutase (Zhang et al., 2010). Glutathione is converted to its reduced form by GR using NADPH. Catalase doesn't require NADPH for conversion of H_2O_2 to water, instead used it for binding to an allosteric binding site that keeps it active in its conformation. SOD also does not require NADPH directly for its catalytic activity but if NADPH is not properly reduced by glutathione system or catalase, increased peroxide levels will eventually inhibit SOD. Decrease in G6PD activity has been recorded in diabetes in brain, liver, RBCs, kidney, endothelial cells and other cells and tissues (Zhang et al., 2010).

2.7.5.7 Uric acid: Many studies have pointed at the prospective involvement of serum uric acid levels with metabolic syndrome and hyperinsulinaemia (Choi and Ford, 2007; Feig et al., 2008). Still, the role played by UA in prognosis of patients with DM, pathophysiology and complications remains unclear. UA values in T2DM patients have been found to be higher (Fang and Alderman, 2000), unchanged (Mohan et al., 1984) or lower (Alderman et al., 1999) as compared to non-diabetics and various factors like gender, glycaemic control and menopausal status have been held responsible for these (Ioachimescu et al., 2007). Studies have shown that T2DM subjects with elevated uric acid values are more prone to developing diabetic co-morbidities especially

cardiovascular (Zoppiniet al., 2009) and renal (Bo et al., 2001; Rosolowsky et al., 2008). It is a strong endogenous antioxidant (Ames et al., 1981; Kalzny et al., 1996) and scavenges nitric oxide (Gersch et al., 2008) thereby decreasing the availability of nitric oxide in vascular muscles and endothelial cells. This leads to endothelial dysfunction increasing the risk of development of CVD (Conen et al., 2004; Feig et al., 2008). In T2DM, hyperuricaemia is connected with impaired glucose tolerance, insulin resistance and early development of nephropathy (Bo et al., 2001). Hyperuricaemia is also associated with components of metabolic syndrome (Oda et al., 2009). A study has shown correlation between serum uric acid levels and age of diabetic patient which exhibits that serum uric acid levels increase with advancement in age (Causevic et al., 2010). Serum uric acid is linked with oxidative stress and generation of tumor necrosis factor (TNF-α) (Butler et al., 2000) which in turn is related to diabetes progression. It is generally considered an end product of purine metabolism which is metabolically inert and of no considerable physiological significance.

2.7.5.8 Creatinine: Serum creatinine is a metabolite formed from creatine, most of which resides in skeletal muscles. The load of creatine per unit mass of skeletal muscles is fixed and is degeneration rate is also constant. Hence, creatinine concentration in plasma is also stable and is a mirror image of skeletal muscle mass (Martin, 2003). Creatinine abandons muscle and circulates in blood whereby it is removed by kidneys. Elevated serum creatinine levels indicate decline in kidney function. Using serum creatinine as a Glomerular filtration rate (GFR) marker was initiated from Rehberg's work in 1926 (Rehman et al., 2005). Creatinine is a sensitive demonstrator of renal function and shows little variation except in case of renal malfunction (www.diabetescurehelp.org/diabetes-tests/bloodurea- nitrogen-tests-17k). Diabetic nephropathy involves a particular set of structural as well as functional kidney

deformities in diabetic patients. Kidney hypertrophy, enhanced thickness of glomerular basement membrane, tubular atrophy and interstitial fibrosis constitute the structural abnormalities while functional aberrations involve escalated glomerular filtration rate with intra glomerular hypertension followed by proteinuria and gradual loss of renal function (Ayodele et al., 2004).

2.8 Metabolic Syndrome (MS) and T2DM

In 1988, Reaven in his review "Role of Insulin resistance in human disease" hinted at insulin resistance to be the common culprit responsible for a syndromic congregation of metabolic risk factors (Reaven, 1988). Thus, the term Metabolic syndrome was born which was later established by World Health Organization (WHO) in 1999 (WHO, 1999). Various other terms have also been given to the syndrome namely, Reaven's syndrome, Deadly Quartet, Insulin Resistance syndrome, Civilization syndrome, CHAOS, Syndrome X and New World syndrome (Joshi, 2003).

Its chief components are obesity, glucose intolerance, hypertension and dyslipidaemia which are major determinants of cardiovascular disease and type 2 diabetes and studies related to it are increasing at a fast pace in various research institutions around the world (Cameron et al., 2004; Davey et al., 1998; Pekkanen et al., 1995). The reason behind is a close-knit interaction among these factors which is associated with the risk of cardiovascular diseases (Borch-Johnsen, 2007). Diabetologists see the syndrome in the light of insulin resistance, cardiologists regard it as dyslipidemia and syndrome X whereas a gynecologist considers it as polycystic ovarian syndrome (Joshi, 2003). Hence covering all the facets of the syndrome which has always lacked a formal structure is a challenge to the research fraternity.

Individuals diagnosed with MS have 30-40% chances of developing T2DM and/or CVD within 20 years depending on the quantitative presence of the factors (Enas et al., 2007).

Each risk factor has its variability attributed to various societal trends such as socio-economic status with rising incomes, mechanization, migration pattern, rural-urban divide, rapid nutritional changes, enhanced education profile and better health care opportunities etc (Misra and Khurana, 2009; Prasad et al., 2010; Vikram et al., 2006). The total risk spectrum of the syndrome is broader than the sum of risk attached to each of the individual components (Afsana et al., 2010, Thorn et al., 2005).

Prevalence of T2DM in developing countries is increasing at an alarming rate culminating in an enhanced burden on the health care services (Shaw et al., 2010). MS if present along with T2DM is said to decrease the survival rate of the patient by 10 years (Hanley et al., 2004). Approximately one third of urban south Asians are evidently suffering from MS (Misra and Khurana, 2009).Yet few studies on MS have been reported from India as compared to other Asian Indian studies out of India (Misra and Vikram, 2002). Thus, research in metabolic syndrome offers a multitude of opportunities to explore the contribution of individual risk factors as well as their combined interplay thereby presenting a holistic view of the condition.

2.8.1 Diagnosis of Metabolic syndrome

Many definitions for the syndrome have been given from time to time, the main points of each are summarised below-

The WHO definition of 1999 (Alberti et al., 1998) includes impaired glucose tolerance or diabetes and or insulin resistance as the mandatory criteria as well as two or more of the following components:

1. Elevated arterial blood pressure ≥140/90 mmHg.
2. Raised plasma triglyceride (≥150 mg/dl).
3. Low HDL-cholesterol, (<35 mg/dl for men and <39 mg/dl for women).

4. Central obesity (WHR: >0.90 for men and >0.85 for women) and/or BMI (>30 kg/m2).

5.Microalbuminurea (urinary albumin excretion rate ≥20 min or albumin: creatinine ratio ≥30 mg/g).

2.8.2 NCEP, ATP III criteria

According to the NCEP, ATP III criteria in 2001 (National Cholesterol Education Program, NCEP, 2001) for aperson to be defined as having the metabolic syndrome she/he must have combination of any three or more ofthe following parameters:

1. Waist circumference (>102 cm for men and >88 cm for women);

2. Plasma triglycerides (>150 mg/dl);

3. HDL cholesterol (40mg/dl for men and 50mg/dl for women);

4. blood pressure (130/85 mmHg), and

5. Fasting plasma glucose (110 mg/dl)

2.8.3 New IDF criteria

According to the IDF definition in 2005 (International Diabetes Federation, 2005) for a person to be defined as having the metabolic syndrome she/hemust have central obesity with ethnicity specific values for different groups as well as two or more of the following criteria.

1. Raised TG levels ≥150 mg/dl (1.7 mmol/l), or specific treatment for this lipid abnormality;

2. Reduced HDL-cholesterol <40 mg/dl (1.03 mmol/l) in males and <50 mg/dl (1.29 mmol/l) in females, orspecific treatment for this lipid abnormality;

3. Raised blood pressure: systolic BP ≥130 or diastolic BP ≥85 mmHg or treatment of previously diagnosedhypertension; and

4. Raised fasting blood glucose ≥100 mg/dl (≥5.6 mmol/l) or previously diagnosed diabetes.

2.8.4 Harmonized definition

According to the harmonized definition given by Joint Interim Statement (JIS) of International Diabetes task force on Epidemiology and Prevention; National Heart, Lung and Blood Institute; World Heart Federation; American Heart Association; International Association for the study of Obesity International Atherosclerosis Society and also Consensus statement for diagnosis of Obesity, Abdominal Obesity and the Metabolic Syndrome for Asian Indians (Alberti et al., 2009; Misra et al., 2009), the diagnostic criteria for MS are-

1. Increased waist circumference (males ≥ 90 cm and females ≥ 80 cm),
2. Hypertriglyceridemia (TG ≥ 150 mg/dl), low HDL (males < 40 mg/dl and females < 50 mg/dl),
3. Elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg or under drug treatment for hypertension)
4. Elevated blood sugar (fasting blood sugar ≥ 100 mg/dl or under treatment for diabetes mellitus).

Any three of the above mentioned conditions present together confirm the presence of MS.

2.9 Trace Metals

Human body harbours an extensive system for management and regulation of the quantity of important trace metals in blood circulation as well as stored in cells. When their concentration in blood is depleted, these nutrient metal ions are secreted in blood from our diet or transported inside cells if their cellular levels are diminished. Moreover, when blood as well as cell is overloaded with them, these are excreted from

the body. When this homeostasis is disturbed, abnormal concentrations and ratios of these trace metals can arise (Kaslow).

2.9.1 Copper

Copper ranks third in the list of most abundant micronutrients in the body (Pedrosa et al., 1999). It can perform the dual function of being a pro-oxidant as well as antioxidant.In the role of antioxidant, it forages upon free radicals and may lessen their impact or aid in preventing their detrimental effects on body (Araya et al., 2006; Bonham et al., 2002; Davis, 2003; Rakel, 2007). When bound with ceruloplasmin, it acts as a prooxidant and gives rise to hydroxyl ions via fenton's reaction (Cunningham et al., 1995). In its unbound or free form, it leads to redox imbalance and initiates intracellular signalling pathways that are stress sensitive via Haber weiss reaction (Cunningham et al., 1995; Mateo et al., 1978). Deranged values of serum copper ions have been reported in studies involving diabetic subjects (Kinlaw et al., 1983). The elevated copper ion levels in diabetic patients are repercussions of hyperglycaemia mediated glycation that releases copper ions accelerating the oxidative stress in body which in turn gives rise to advanced glycation end products that are connected with the pathophysiology of diabetic co-morbidities (Mosad et al., 2004). In plasma, copper mainly binds with albumin and ceruloplasmin and it has been reported that long standing hyperglycaemia can destroy the affinity of both the proteins towards copper (ArgirovaandOrtwerth, 2003). It is vital for the catalytic action of Superoxide dismutase (SOD) that scavenges superoxide radicals (Olivares et al., 2000). Role of copper imbalance is suspected in the elevation of cholesterol by disruption of normal HDL (High density Lipoprotein) and low LDL (Low density Lipoprotein) balance (Jackson et al., 2010). A clinical study conducted among diabetics showed that a person with higher

copper levels was more likely to develop diabetic complications like retinopathy, vascular disease or high blood pressure (Walter et al., 1991).

2.9.2 Zinc

Zinc is intricately involved in cellular metabolism via numerous channels (Classen et al., 2011). Muscles and bones contain about 85% of the total body content of Zn, while 11% resides in liver and skin (Chasapis et al., 2012). Zn is an integral cofactor of more than 300 enzymes and also plays significant part in immune system, oxidative stress, aging and apoptosis (Chasapis et al., 2012). An alteration in metabolism of Zn is one of the contributing factors towards oxidative stress that is inherently involved in the pathogenesis of type 2 diabetes (Cruz et al., 2015). Various studies have hinted at the Zn binding site of matrix metalloproteinases being associated with development of microvascular ao morbidities of diabetes and diabetes tendon disorders (Abreu et al., 2016). It is considered an important factor involved in glucose homeostasis and the risk of developing T2DM (Wijesekara et al., 2009).

Inactive zin crystals are believed to be repository of insulin (Singh et al., 1998). Within the secretory granules of cell, zinc ions aid in forming insulin hexamers which are somatically stable. When secretory granules rise to the surface and open, pH increases to body's physiological levels, causing the pressure of zinc ions to fall steeply resulting in the formation of free insulin monomers whereas zinc ions are expelled from the pancreas (Sondergaard et al., 2003). Thus zinc is inherently involved in insulin synthesis as well as storage (Chausmer, 1998). The invitro effectiveness of insulin has been found to be enhanced by Zn ions and their deficient levels may exacerbate the insulin resistance in T2DM (Arquilla et al., 1978). Moreover, it has been observed in experimental models that zinc deficiency makes the body highly susceptible towards

lipoprotein oxidation (Disilvestro, 2000) which is highly prevalent in T2DM subjects (Davi et al., 2005).

2.9.3 Significance of Cu/Zn ratio

Enhanced copper and diminished zinc is one of the most common imbalances encountered among the trace metals (Kaslow). Copper and zinc both are important for proper functioning of CuZn SOD1 enzyme found in cytosol, nucleus, mitochondria and peroxisomes (Cao et al., 2008). Reduced copper ion concentration may retard the activity of superoxide dismutase and the destructive streak of superoxide radicals continues. Zn ions are coordinated to enzyme Cu/Zn superoxide dismutase and this coordination is tightly regulated by protein's folding pathway. It first catalyses the folding reaction by transiently binding with Cu ions, then eventually shifts to its higher affinity Zn site later. Lifetime of the enzyme is considerably reduced if the dissociated Zn ions are prevented from rebinding to SOD structure (Leinartaite et al., 2010). Thus, while functioning in the enzyme, copper and zinc work together and it is actually their ratio rather than their absolute amounts that helps in the proper functioning of this enzyme (Groff et al., 1995; Harris, 2001).

2.9.4 Iron

Iron is an essential element needed by the body for the production of two vital functional proteins like haemoglobin and myoglobin which aid in transport of molecular oxygen during respiration (Ganz and Nemeth, 2006). It is also involved in production of elastin together with zinc and ascorbic acid and synthesis of collagen (Stechmiller, 2010)

Insulin resistance is synonymous with diabetes and various studies point to its connection with iron overload in body. Iron is a transition metal capable of redox activity and any potential harm targeted at the body is prevented by its binding with

transport or storage proteins (Mccord, 1991). In blood iron is carried by transferrin, a protein made in liver. In its free form i.e., in non-transferrin bound form it is known to induce oxidation of biomolecules through Haber-Weiss and Fenton reactions by producing harmful hydroxyl radicals (Halliwell andGutteridge, 1990). These free radicals are powerful prooxidants which cause lysis of lipid cellular membrane, damage protein structural configuration and displace nucleic acid in genes (Mccord, 1996, Witte et al., 1996). Thus the catalytic action of free iron contributes initially to insulin resistance and later on to reduced insulin release which subsequently results in the development of T2DM (Beard, 2001; Oberley, 1988; Wolff, 1993). Emerging scientific evidence has disclosed that the relationship is bidirectional wherein glucose metabolism also impinges upon several iron metabolic pathways. Inflammatory cytokines in oxidative stress influence these relationships, replicating and intensifying the initiated events. Chronic diabetic complications are also modulated by iron induced damage (Kim et al., 2000).

Increased serum iron levels among general poulation are found in hemolyticanemias, hepatitis, lead and iron poisoning whereas decreased serum levels are found in anemias caused by iron deficiency due to insufficient intake or absorption of iron, chronic blood loss, late pregnancy and cancer. The role of iron in the pathogenesis of T2DM calls for further studies owing to increased incidence of iron overload encountered among diabetics which can be reversed by achieving targets of good glycaemic control using either phlebotomy or iron chelation therapy (Swaminathanet al., 2007).

2.10 Genetic Polymorphism and Diabetes

Single Nucleotide Polymorphisms (SNPs) are variations in DNA sequence that result when a single nucleotide (A, T, G or C) gets altered in the genome sequence (see Figure 2.5). A variation is termed a SNP, if it occurs in at least 1% of the population. SNPs

constitute about 90% of the variations in DNA sequence of human genome. They repeatedly occur about every 100 to 300 bases along the 3 billion bases constituting the human genome. SNPs are easier to track in population studies since they do not change much from generation to generation i.e. evolutionarily stable. These DNA sequence variations can influence the human response towards a disease, environmental adversities such as viruses, bacteria, toxins, drugs, chemicals and other therapies. Many of these SNPs are candidate genes for disease susceptibility and this has given way to numerous case control studies in finding the association between a particular polymorphism and diseases (Roses, 2000). Thus they have emerged as genetic markers of choice owing to their even distribution and high density in the human genomes (Kruglyak, 1999; Sachidanandam et al., 2001; Venter et al., 2001). With the advancement of biotechnology, SNPs are increasingly being used in map based cloning (Wang and Liu, 2006), marker assisted breeding (Flint-Garcia et al., 2003), study of evolutionary conservations between different species (Feltus et al., 2004; Hillier et al., 2007) and detection of risk associated alleles linked to human diseases (Eberle et al., 2007).

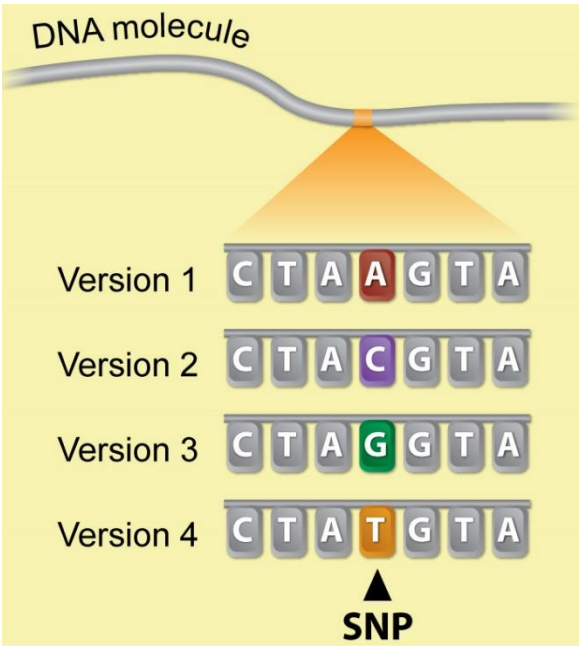


Figure 2.5: Single Nucleotide Polymorphism.
(Adapted from http://learn.genetics.utah.edu/content/precision/snips).

T2DM and the co morbidities attached with it are very common in Indians which diminish the overall quality of life by imposing burden on healthcare (Unnikrishnan et al., 2016). It is a challenge to shed light on the genetic components that participate in the pathogenesis of these complications. Studies focussing on the genetics of T2DM are useful in enriching the existing knowledge and may provide useful links that might allow early disease detection and proper management of co-morbidities if any.

2.10.1 Methods to study SNPs

Researchers have employed plethora of techniques to study SNPs. Some of the widely used techniques are as below-

(a) Restriction Fragment Length Polymorphism (RFLP): It is one of the early methods established and widely used for SNP genotyping. Method works when presence of SNP either abolishes or creates a site for restriction enzymes.

(b) Ligation based assay: Two allele specific oligonucleotides are allowed to anneal with template DNA followed by use of ligase enzyme. Ligation happens only if oligonucleotides display perfect match thereby offers allelic discrimination.

(c) Allele Specific Oligonucleotide (ASO) Hybridisation: Short nucleotide probes corresponding to either wild type or mutant sequence is used under stringent hybridisation conditions.

(d) Allele specific PCR or ARMS (Amplification Refractory Mutation System) PCR: Requires two allele specific primers and one consensus primer. Reaction has to be initiated in two set each corresponding to alternate alleles.

(e) Single nucleotide extension

Figure 2.6 summarizes the basic principle behind these commonly used SNP genotyping techniques.

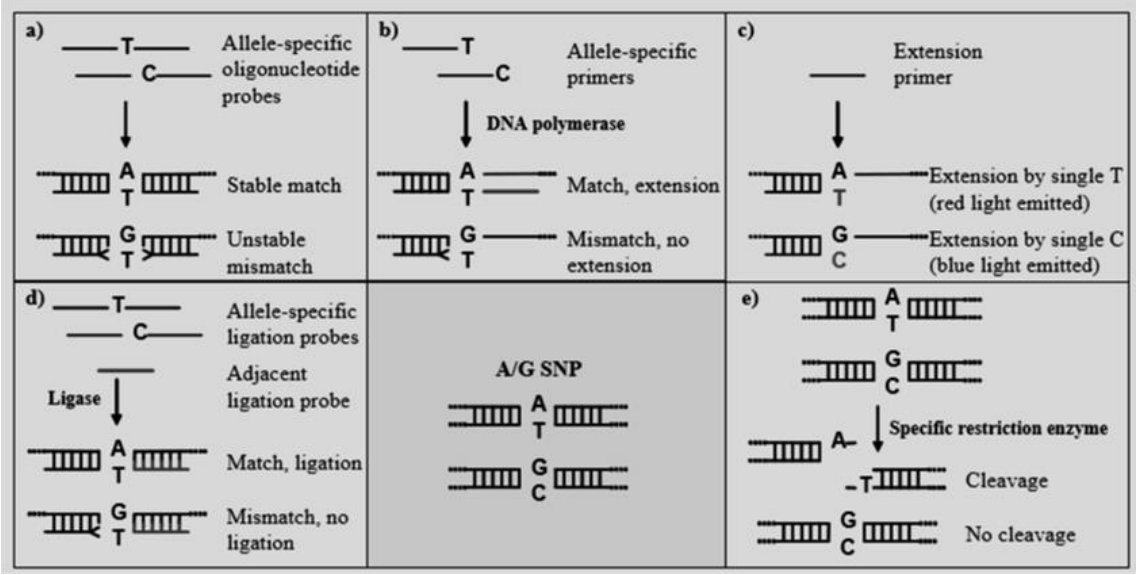


Figure 2.6: Common methods to study SNPs (adapted from Routanen, 2007).

2.10.1 Transforming Growth Factor Beta 1 (TGFß1) gene

The progression of T2DM is influenced by several genetic as well as environmental factors (Ahlqvist, 2011). Exhaustive efforts have been dedicated to the identification of type 2 diabetes related genes for better understanding of its pathogenesis, finding novel targets for therapy and permitting the prediction of disease (Ahlqvist, 2011). Various studies in the last few years were conducted that aimed at studying the effect of pro-inflammatory cytokines. It has been reported that these cytokines play important role in the progression of T2DM and its co-morbidities (Yih- Hsinet al., 2009). Chronic inflammation and disturbed homeostasis of immune system is crucial in the development of T2DM. Pro-inflammatory as well as anti-inflammatory cytokines exhibit differential expression during insulin resistance and other stages of T2DM (Navarro-Gonzalez and Mora-Fernandez,2008).

Transforming Growth Factor beta 1 is an important anti-inflammatory cytokine that helps in averting autoimmune reactions and instigates immune tolerance by impeding macrophage activation (Li et al., 2006). TGF beta 1 gene is located on chromosome 19q13.1-13.3 (Fujiiet al., 1986) and multiple known polymorphic loci are found in exons, introns and the 5' flanking region (Watanabe et al., 2002). Various studies have reported the stimulation and enhanced expression of TGF beta 1 both in vitro and in vivo by hyperglycaemia and hyper-insulinemia (Sarafidisand Ruilope, 2006). A correlation between enhanced TGF beta 1 expression and diabetic nephropathy has been documented (Singh and Ramji, 2006). Diabetic nephropathy is distinguished by accumulation of extra cellular matrix (ECM) and hypertrophy of kidney glomerular cells leading to glomerulosclerosis. These modifications give way to microalbuminaria, albuminaria, uremia and lastly to ESRD (End Stage Renal Disease) (Goldberg, 2009). TGF beta 1 is found to be highly expressed in mesanglial cells of diabetic glomeruli and

disrupting its function leads to prevention of glomerular fibrosis. Anti-inflammatory function of TGF beta 1 as well as its signalling mechanism are rendered inactive by mutation /altered expression during the course of T2DM (Johansson and Grapin-Botton, 2002). Various studies conducted among different populations have pointed out the association between T869C polymorphism and progression of T2DM (Sherifet al.,2013; Teresa et al., 2003). This polymorphism results in proline getting substituted for leucine at 10^{th} amino acid and is generally related to an increase in its expression in vitro (Awad et al., 1998). The secretory function of pro10(C) allele is almost twice that of leu10(T) allele (Dunning et al., 2003). TGF beta 1 over expression is one of the most potent elements of tissue fibrosis culminating into organ failure (Border and Noble, 1994). Thus, the T allele seems to of protective nature while C allele is the susceptible culprit behind T2DM occurrence (Sherif et al., 2012). A recent Indian study has reported that the CC genotype of this polymorphism elevates the risk of nephropathy 3.1 to 4.5 times in Indian population (Raina et al., 2015).

2.10.2 MTHFR gene

Methylene tetrahydrofolate reductase (MTHFR) is a crucial part of folate metabolism. It is a vital gene of remethylation pathway and an enzyme dependent on vitamin B12. The gene resides at 1p36.3. It catalyses the reduction of 5'10 methylenetetrahydrofolate reductase, which is the principal circulating form of folate and also a carbon donor for remethylation involving a series of reactions that transform homocysteine to methionine (Saffari et al., 2013). Methionine formed is important for synthesis of proteins and other vital compounds by the body (Wang et al., 2013). Diminished function/amount of the enzyme causes homocysteine levels to shoot up in blood. These enhanced homocysteine levels are shown to be a risk factor for atherosclerosis (hardening of the arteries), which eventually leads to a heart attack and venous thrombosis (Elizabeth et al., 2005). There

are two common polymorphisms associated with MTHFR gene i.e. C677T and A1298C. The former found in exon 4 of MTHFR gene, resides within the NH2-terminal catalytic domain while the latter lies in the COOH-terminal regulatory region of exon 7 (Pandey et al., 2011). The A1298C (rs1801131) polymorphism is synonymous to decreased activity of the enzyme invitro (Van der put et al., 1998, Weisberg 1998, Naomi 2001). The SNP consist of replacement of A by C at neucleotide 1298 in exon 7 resulting in a glutamate to alanine substitution (Jakubowski, 2000).

While many studies have been conducted on C677T, which is the most prevalent polymorphism associated with MTHFR gene, lesser information is available regarding variant A1298C. Both these SNPs result in diminished enzyme activity (Castro et al., 2003). The frequency of these two mutations and alterations in enzyme activity vary among different ethnic populations and geographical regions (Esfahaniet al., 2003, Yang et al., 2013). Some studies have linked this hyperhomocysteinemia with insulin resistance (Scullion et al., 2012) which is one of the prime cause of diabetic co-morbidities such as diabetic nephropathy (Mitraoui 2007; Ukinc et al., 2009). Oxidation of homocysteine may cause generation of hydrogen peroxide and free radicals, enhancing the oxidation of LDL thereby causing damage to the endothelial lining, which is a crucial component of atherosclerosis (Heinecke et al 1987; Parthasarathy, 1987; Starkenbaum and Harlan,1986). Various studies have shown the association between MTHFR polymorphism and risk of T2DM (Abdraboh et al., 2013; Settin et al., 2015; Zhang et al., 2014) While other studies have found no significant association between these polymorphisms and T2DM patients of Asian, Caucasian and African descent (Mitraouiet al., 2007, Zhong et al., 2013).

2.10.3 PTPN22 gene

Protein tyrosine phosphatise non- receptor type 22(PTPN22) located on chromosome 1p13.3-p13.1 encodes for the lymphoid tyrosine phosphatise (LYP). LYP is a 105 KDa protein which greatly suppresses the process of T- cell activation as well as T- cell proliferation. Investigations based on genetics and immunology studies have highlighted the role of these in type 1 diabetes development and progression. Alterations in signalling pathways within various cell types, causing central as well as peripheral tolerance failures, advancement of pro inflammatory T cell responses and loss of glucose homeostasis regulation have been unravelled by the study of these phosphatases (Cerosaletti and Buckner, 2013). Recently, a functional SNP at nucleotide 1858 in codon 620 of PTPN22 (1858C >T; rs2476601 R620W) has been reported to be associated with T2DM (Cervin et al., 2008; Douroudis et al., 2008).

2.11 Metabolomics

Type 2 diabetes is hardly a static phenomenon. It evolves and transforms over the time during a patient's life span. Moreover, the effect of disease varies from person to person (Grundy et al., 2012; Lui et al., 2007; Rosenzweig et al., 2008). Appreciable variation is observed in the response of patients along with their susceptibility to diabetic co morbidities. Such variegation in disease development as well as treatment outcome highlights the demand for supplementary mechanisms for forecasting disease development as well as favourable treatment results. Metabolomics is considered a novel approach in achieving these targets. It involves exhaustive signalization of metabolites in biological setup. The metabolome encompasses various small arbitrator molecules and metabolic products released with energy consumption and storage, carbohydrate and protein precursors, modulators of gene expression as well as signalling molecules. Thus, metabolome represents a real time effective snapshot of the

cell or organism. It is affected by multiple factors like lifestyle, diet, age, medications and gender. It employs various powerful techniques and Nuclear Magnetic Resonance (NMR) is one of them (Zacharias et al., 2013). NMR technique is broadly based on the magnetic properties of the atomic nucleus. It was used for the first time in 1980's for body fluid's analysis (Bell et al., 1989; Iles et al., 1985, Nicholson et al., 1984). Structural and chemical properties of a molecule are determined on the basis of the behaviour of NMR active nuclei in the magnetic field. Since ^{1}H nucleus are plentiful, investigations pertaining to body fluids like plasma and urine as well as tissue relies heavily on ^{1}H NMR spectroscopy. Most common samples in NMR experiments are serum, plasma (Klein et al., 2014), and urine (Zacharias et al., 2013; Zhang et al., 2014). Other sample types used are cerebrospinal fluid (Trushina et al., 2013) and saliva (Mook-Kanamori et al., 2014). Some studies have also employed tissue extracts (R¨omisch-Margl et al., 2012); whole tissue (Beckonert et al., 2010) and even living organisms (Feyter et al., 2013) for analysis using different variants of the technique. Cell culture samples and supernatants may also be used (Wallace et al., 2013).

This technique has been applied for measuring lipoprotein particles in clinical samples for more than a decade (Klein et al., 2014; Zacharias et al., 2013; Zhang et al., 2014). A change has also been observed in the amino acid profile of T2DM patients (Lanza et al., 2010; Messanaet al., 1998, Suhreet al., 2010), which underlines the influence of amino acids on insulin action and subsequently glucose metabolism.

NMR technique enjoys a great range of simultaneous detectable metabolites not to mention a simple sample preparation and excellent reproducibility and non-destructive nature of analysis (Klein and Shearer, 2015). Apart from diagnosing the onset of T2DM, another area for metabolomics research is disease prognosis and its outcome Kafter onset. T2DM in its course is develops many co-morbidities like cardiomyopathy,

nephropathy, neuropathy and retinopathy. Little metabolomics research has been attempted in this area on human subjects. Improved prognosis may encourage early treatment interventions and reduce T2DM burden. Till now, most studies involving metabolomics of T2DM have focussed on its prediction rather than its co morbidities. Thus, combining metabolomics data with clinical and molecular data may prove to be valuable in prediction of T2DM as well as its co morbidities.

2.12 Diabetes Management: Prevailing Issues and Preventive Measures

2.12.1 Social stigma attached with diabetes

Proper management of diabetes in developing countries faces many check points. Social barrier is one of them. Youngsters newly diagnosed with diabetes are not forthcoming about their disease status and do not pursue treatment /medication. This may be due to fear of being turned down in jobs or marriage proposals (Roglic et al., 2005). Proper care and management in such cases is tough and advising such patients to comply with the treatment is challenging. Dearth of regular monitoring and awareness about the disease further adds to the dilemma. Furthermore, from diagnosis to management the Indian diabetes scenario is dominated by general physicians rather than specialised diabetologists. Thus general physicians have dual responsibility of updating their knowledge about different aspects of diabetes from time to time and monitoring and counselling of the patients to reduce the development of co-morbidities (Viswanathan and Rao, 2013).

2.12.2 Financial burden of diabetes

Diabetes is a costly affair to deal with. A study in India reported that diabetes care cost varied from 1230 billion to 1837.3 billion Indian rupees in 2010 (Tharkar et al., 2010). Co morbidities if present further raises the treatment costs, for ex., charges for care for a

diabetic patient with foot ulcers was four times of what a diabetic without foot ulcer had to pay (19020 rupees in former as compared to 4493 rupees in the latter) (Satyavaniet al., 2012). There are also hidden or indirect economic losses owing to lost workdays/economic opportunities. Furthermore, with no feasible social security network in our country, a patient becomes the responsibility of his/her family for support. Thus, if an earning member is affected from diabetes, resources of the family take a hit which may also lead to extended financial consequences for them (Kapur, 2007). Tharkar et al., reported that greater than 60% of the people belonging to low income strata were compelled to acquire loans or mortgage property for their diabetes treatment and about 70-80% of patients belonging to economically well off class used their savings for their treatment (Tharkar et al., 2010). Hence, people with restricted economic means are forced to disburse their funds on management of diabetes (Ramachandran et al., 2007). The incidence of co morbidities further exacerbates the situation. A study conducted in south India documented the hike in expenses borne by the patients with diabetic co morbidities in rural us well as urban localities (Ramachandran et al., 2007).

2.12.3 Prevention of diabetes

Prevention is better than cure. This age old saying aptly suits the basic principles of diabetes management. Imparting proper knowledge at community level especially to at risk population about diabetes and ensuing complications is an important preventive measure. A constructive educational- cum- awareness program stressing on healthy diet, physical activity, abstinence from smoking and drinking and stress free life could prove to be productive in minimizing the impact of diabetes. Giving proper education to every patient visiting a diabetes clinic or hospital should be the norm. In a country like ours

which is battling with issues like unemployment and slowly rising out of poverty, the execution of the above mentioned plans is not very pragmatic. In this scenario, the role of healthcare organizations becomes important in encouraging diabetes education and management through awareness campaigns at community level (Viswanathan and Rao, 2013).

2.12.4 Diabetes education programs in India: an overview

The Prevention, Awareness, counselling and Evaluation (PACE) diabetes project

It was a landmark study conducted at Chennai, south India to spread awareness about diabetes, associated complications and risk factors among the local population of Chennai city (Kapur et al., 1997). It was done via media campaigns, public education, training of general physicians and community based prevention schemes. It led to a significant increase in diabetic knowledge among the people regarding complications and risk factors like hypertension, family history, obesity and stress. Overall, the program led 46% to realize that diabetes is preventable (Somannavar et al., 2008).

Another study involving the use of SMS (Short Messaging Service) to provide knowledge to patients regarding drug usage, dietary modifications, increased physical activity and motivating them to comply with them was received well with the patients (Shetty et al., 2011). Significant decrease in the glycaemic levels of patients who received the message was observed as compared to those patients who received similar care excluding the messages (Shetty et al., 2011).

A national programme for prevention and control of diabetes, cardiovascular disease and stroke had been launched by the National Rural Health Mission which offers opportunities for betterment of diabetes care at primary and secondary level care centres (National Rural Health Mission, 2005).

ICMR and WHO have jointly developed the guidelines for diabetes management in the Indian context (ICMR, 2005). Many community-based NCD projects are aimed at preventing diabetes via lifestyle modifications, weight control and use of prescribed drugs have shown a decrease in risk factors upon following a healthy lifestyle (WHO SEARO, 2003) and maintaining optimum weight (BMI: 18.5-24.9 kg/m^2) (Yadav et al., 2008) which is beneficial to the society at large.

CHAPTER 3:

MATERIAL AND METHODS

MATERIAL AND METHODS

3.1 Selection of study subjects

The type 2 diabetic patients (n=120) were enrolled from those attending the OPD of GSVM medical college and hospital and some private clinics under the supervision of expert clinicians. Age and sex matched normal and healthy individuals (n=120) were screened from students and members of the university, their family members, relatives and departmental staff. The study was conducted after due approval from institutional ethics committee (G.S.V.M. Medical College, ethical code No.14/Steno, on 13 January 2011) and written informed consent was obtained from all the study subjects.

3.1.1 Inclusion criteria

Controls- Asymptomatic individuals.

Patients- Type 2 diabetes patients who were medically stable for 60 days from surgical procedure or other significant illness (including diagnosed diabetes).

3.1.2 **Exclusion criteria**

The exclusion criteria for patients extended to those diagnosed with type 1 DM, acute complications such as severe infections or major operations, trauma, history of alcohol abuse, severe cardiovascular/respiratory diseases, and pregnant and breast feeding women. Newly diagnosed cases and those suffering from chronic diabetic co-morbidities were also excluded from the study.

3.2 Anthropometric features

Obesity guidelines following western populations distinctly underrate the risk among Asians because they display greater body fat at a given BMI. For BMI cutoff values we referred to consensus guidelines (WHO, 2000) for Asian Indians i.e. normal (BMI 18.5-22.9 Kg/m^2), overweight (BMI 23-24.9 Kg/m^2) and obese (BMI $\geq$ 25 Kg/m^2).

3.2.1 Calculation of BMI:

$$BMI = (\text{weight in kilograms}) / \text{height in meters}^2$$

Blood pressure was measured using standard electronic B.P. measuring device.

3.2.2 Questionnaire based analysis

Information regarding patient history, socio-economic and demographic features, disease awareness and treatment adherence was obtained by a personal interview of all the patients using a structured health assessment questionnaire (HAQ). The questionnaire was developed and suitably modified after consultation with experts.

The classification of various socio-economic features is as follows-

3.2.2.1 Physical Activity

Mild- Slow walking, light cleaning, other activities which mostly involve sitting and driving for most of the day were characterized as mild physical activity.

Moderate- Lots of walking and other activities that involve moving for several hours in a day qualified as moderate physical activity.

Vigorous- Highly active/athletic lifestyle, heavy manual labor, dancing or playing of an active sport for several hours a day constituted vigorous physical activity.

3.2.2.2 Smoking

For analysis purpose, ex as well as current smokers were categorized in the "ever smoker" group which was kept under "yes" category in the table whereas non- smokers were kept in "No" category.

3.2.2.3 Alcohol consumption/Drinking

Subjects who had consumed alcohol at least once in the last month were defined as current alcohol users and were kept under "Yes" category and rest under "No" category.

3.2.2.4 Consumption of junk food

Frequently- Subjects who enjoyed junk food at least once in a week.

Sometimes- Subjects who enjoyed junk food at least once in a month.

Rarely- Subjects who abstained from junk food for long times (more than a month) came under this category.

3.2.2.5 Economic status

LIG (Low Income Group) - income of ≤ 2500 Rs per month

MIG (Middle Income Group) – income of 2500-25,000 Rs per month

HIG (High Income Group) – income >25,000 Rs per month

3.3 Biochemical estimations

3.3.1 Collection of blood, plasma, and RBC

Fasting heparinized 5.0ml blood of subjects were collected from the median vein of the forearm. 1.0ml blood was collected in fluoride vial (KF) for plasma glucose estimation. Rest 4.0ml blood without delay was centrifuged for 10 minutes at 15,000 rpm in refrigerated centrifuge machine (0-5ºC) so as to collect plasma and plasma free Packed Cell Volume (RBC etc.) and processed immediately for preparation of hemolysate. For collection of serum, plain vial was used.

3.3.2 Preparation of hemolysate

Plasma removed PCV (Packed cell volume) was washed 3 times with normal saline by the repeated centrifugation for 5 minutes at 15,000rpm a 0-5^{0}C at each wash. Cells were lysed by adding 1.0 ml chilled distilled water for 10 minutes, and then were shaken vigorously for 2 minutes, 0.5 ml of chloroform was added as preservative. Mixture was centrifuged at 3000 rpm for 20 minutes. Mixture was clearly separated into 3 layers, lower most layer was of chloroform, middle of cell stroma (mucus) and upper most layer was of clear hemolysate. Estimation/determination of each parameter under study was completed same day and hemolysate, plasma was preserved at 0 - 4°C when it was not in use.

3.3.3 Methodology and estimation protocol of parameters under study

3.3.3.1 Plasma glucose levels

Glucose estimation was done by the enzymatic Kit method of GOD/POD (Trinder, 1969).

Reagents required/prepared: -

1. Reagent 1: Glucose reagent (phosphate buffer, glucose Oxidase/peroxidise, 4-AAP and stabilisers).
2. Reagent 2: Glucose Diluent (Phenol preservative)
3. Working Reagent: 1 vial reagent 1 + 50ml reagent 2
4. Glucose standard: Dextrose in preservative (100mg/dl).

Procedure:

Preparation of Blank: working reagent 1500µl

Preparation of Standard: working reagent 1500µl + glucose standard 20µl

Preparation of Test: working reagent 1500µl + plasma 20µl

Common procedure:

1. Mixed well all the three (blank, standard and test), incubated at 37°C for 10 minutes or at room temperature (15° - 30°C) for 30 minutes.
2. Added triple distilled water in all the test tubes
3. Measured the standard followed by test at 505nm.

Calculation:

$$\text{Plasma glucose (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

3.3.3.2 Glycated haemoglobin estimation

Glycated haemoglobin (HbA_1C) estimation was done by the modified colorimetric method of Fluckiger, R. and Winter halter, K.H. (1976).

Reagents required/prepared: -

1. 0.3% oxalic acid w/v
2. 40% T.C.A. (Trichloro-acetic acid) w/v
3. 0.05 M thiobarbituric acid

Test for glycated haemoglobin:

To 2 ml of haemolysate, 1ml of 0.3 N oxalic acid was added and heated in boiling water bath at 100°C for 60 min. After cooling 1 ml of 40% T.C.A. was added, shaken vigorously and centrifuged. To 2 ml of this supernatant, 0.5 ml of 0.05 M thiobarbituric acid was added and incubated at 37° C for 30 min.

The resultant yellow colour was read at 443 nm.

Calculation:

HbA1c was calculated on assumption that 1% HbA1c corresponds to an absorbance of 0.029 at 443 nm.

3.3.3.3 Lipid Profile

3.3.3.3.1 Estimation of serum total cholesterol

Span diagnostic kit was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method.

Principle

The enzyme, cholesterol esterase catalyzed hydrolysis of cholesterol esters to free cholesterol and fatty acid molecules. Then free cholesterol gets oxidized in the presence of cholesterol to form cholest -4en-3-one and H2O2. Liberated H2O2 reacts with

phenol and 4 AAP in presence of peroxidase to form red coloredquinoneinine complex the intensity of which was measured at 505 nm 130,131.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesteryl ester hydrolase}} \text{cholesterol} + \text{Fatty acid}$$

The 3- OH group of cholesterol is then oxidized to ketone in oxygen requiring reaction catalyzed by cholesterol oxidase.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholesten-3-one} + H_2O_2$$

H2O2one of the reaction products is measured in a peroxidase catalyzed reaction that forms a dye.

$$2\,H_2O_2 + \text{Phenol} + 4\,\text{AAP} \xrightarrow{\text{Peroxidase}} 4\,H_2O + \text{Quinoneimine dye}$$

3.3.3.3.2 Estimation of serum of triglycerides

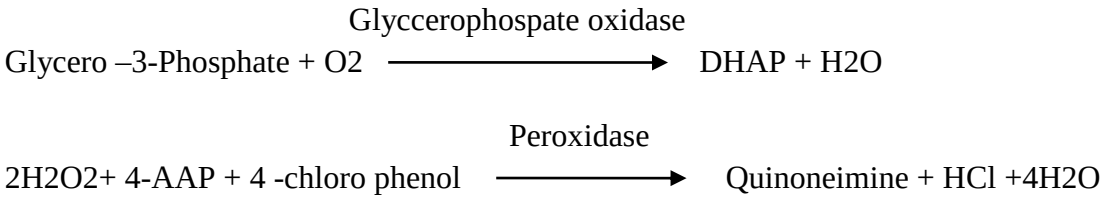
Span diagnostic kit was used for estimation of triglycerides, which followed end point colorimetric enzymatic test using glycerol-3-phosphate oxidase**.**

Principle:

The enzyme, lipoprotein lipase catalyzes hydrolysis of TGs to glycerol and FAs. Glycerol then is phosphorylated in an ATP -requiring reaction catalysed by glycerophosphate. The formed glycerophosphate is oxidized to dihydroxyacetone and H2O2in a glycerophosphate oxidase catalyzed reaction. H2O2then reacts with 4 -AAP and 4 -chlorophenol under the catalytic influence of peroxidase to form colored quinoneimine complex, the intensity of which was measured at 505nm.

$$\text{Triglyceride} + 3H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3\ \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycero –3-phosphate} + \text{ADP}$$



3.3.3.3.3 HDL cholerterol

It was estimated by PEG Precipitation method using colorimetric kit from Coral clinical systems (Goa, India).

Reagents required:

L1: Precipitating reagent- 10 ml

L2: Enzyme reagent

S: HDL Cholesterol standard (mg/dl)- 5 ml

0.5µl of precipitating (L1) reagent and 0.5 ml of serum/plasma was mixed well and incubated at room temperature for 10 min. and then centrifuged at 3000 rpm to obtain a clear supernatant.

Addition sequence	**Blank**	**Standard**	**Test**
Enzyme reagent (L2)	1 ml	1 ml	1 ml
Standard	-	10 µl	-
Supernatant	-	-	10 µl

The reaction mixture was mixed well and incubated for 5 min. at 37^{o}C. The absorbance was measured at 510 nm.

Calculations:

HDL cholesterol mg/dl = (Abs. of test/Abs. of Standard) *200

Normal range:

Male- 30-60 mg/dl

Female- 40-70 mg/dl

3.3.3.3.4 LDLcholesterol

LDL cholesterol was calculated by using the formula

LDL cholesterol=Total cholesterol –HDL cholesterol –triglyceride

LDL cholesterol level in plasma was expressed as mg/dl.

3.3.3.3.5 VLDL Cholesterol

VLDL Cholesterol was calculated by the formula

VLDL Cholesterol= Triglyceride/5

3.3.3.4 Uric Acid

It was estimated by colorimetric method using kit from Tulip diagnostics.

Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

NORMAL RANGE

Serum / Plasma (Males): 3.4 - 7.0 mg/dl

(Females): 2.5 - 6.0 mg/dl

Reaction contents 75 ml

L1: Buffer Reagent- 60 ml

L2: Enzyme Reagent- 15 ml

S: Uric Acid Standard (8 mg/dl)- 5 ml

Working reagent: 0.8ml of L1 and 0.2 ml of L2 was used as the working reagent during the assay.

Assay

Addition Sequence	Blank (ml)	Standard (ml)	Test (ml)
Working Reagent	1.0	1.0	1.0
Distilled water	0.02	-	-
Uric Acid Standard	-	0.02	-
Sample	-	-	0.02

Reaction mixture was mixed well and incubated at 37^{o}C for 5 min. and absorbance of standard (Abs.S), test sample (Abs.T), was read against blank within 30 min at 546 nm.

Calculation:

Uric Acid (mg/dl) = (Abs.T/Abs.S)*8

3.3.3.5 Creatinine

Picric acid in an alkaline medium reacts with creatinine to form an orange coloured complex with the alkaline picrate. Intensity of the colour formed during the fixed time is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline Picrate Orange Coloured Complex

Normal Range (Serum/Plasma):

Males: 0.6-1.2 mg/dl

Females: 0.5-1.1 mg/dl

Reagents (2X75 ml kit)

L1: Picric Acid Reagent- 75 ml

L2: Buffer Reagent- 75 ml

S: Creatinine Standard (2mg/dl)- 5 ml

Working reagent- It was prepared by mixing equal volumes of Picric acid reagent and Buffer reagent.

Assay

To the tubes marked test/standard, 0.5 ml L1 reagent and 0.5 ml L2 reagent was added. 0.1 ml of standard was then added to the tube marked as standard and 0.1 ml of serum was added to the tube marked as test. Reaction mixture was mixed well and read the initial absorbance A1 for standard and test after exactly 30 seconds. Second absorbance A2 of standard and test was read exactly 60 seconds later.

Calculations:

For standard: $S = A_2S - A_1S$

For Test: $T = A_2T - A_1T$

Creatinine (mg/dl) =

3.3.3.6 MDA (Malonaldehyde) Level

Serum MDA levels was estimated as per the method of Satoh (1978). 200 µL of serum was added to 300 µL of trichloroaceticacid (TCA)-thiobarbituric acid (TBA)-Hydrochloric acid (HCl) solution. The composition for the TCA-TBA-HCl solution was 15% (w/v) TCA, 0.375% (w/v) TBA and 1M HCl, which were mixed in equal volumes. The reaction mixture was incubated in a boiling water bath for 10 minutes. After incubation, the reaction mixture was cooled completely and 500 µL of Centrifugation at 2000 rpm at 15 °C was carried out for 20 minutes. 100 µL of protein free-supernatant was pipetted out and 25 µL of 1 M sodium hydroxide was added to eliminate the white precipitate formed. Normal saline was used instead of serum as blank. The absorbance of the reaction mixture was measured at 535 nm, and results were expressed as µmol/L/mg Protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

MDA (µmol/l) = OD_{532} X 1.75 / 0.156

$O.D._{532}$ (optical density in λ) =532 nm and extinction = $1.56 \times 10^5\ M^{-1}\ cm^{-1}$

3.3.3.7 Catalase activity assay (method of Ashok K. Sinha, 1972)

Reagents required are Dichromate/acetic acid. (5% potassium dichromate (w/v) 50 ml + 98 – 100% glacial acetic acid (w/v) 150 ml, Hydrogen peroxide (0.2M) solution, Phosphate Buffer (0.01M), pH 7.0

Sample prepration

Haemolysate was diluted with 20 parts of cold distilled water and it was used as such. 4ml of H_2O_2 solution was taken in a small beaker in which 5ml of phosphate buffer was added, 1ml of properly diluted sample (haemolysate) was mixed rapidly with gently swirling motion. 2ml of dichromate/acetic acid reagent was taken in test tubes labeled 1,2,3,4 1ml of reaction mixture made previously in a small beaker was withdrawn and added to the test tubes containing dichromate/acetic acid reagent at an interval of 60 second for each tube. O.D. at 570nm of four tubes (1,2,3,4) was read.

Standard curve preparation

Different amount of H_2O_2 ranging from 40 - 160µmoles was taken in small test tubes in increasing order of concentration (like 40, 80, 120 & 160µmoles). 2ml of dichromate acetate was added to each and unstable blue precipitate of perchromic acid was obtained. It was heated for 10 minutes in a water bath, which changed colour of solution to green, due to formation of chromic acetate. It was cooled at room temperature and volume of the reaction mixture was made to 3ml by distil water. O.D. was read at 570nm and standard curve was plotted between O.D. and amount of H_2O_2.

The activity of catalase was expressed as µmoles of hydrogen peroxide consumed/min/gm Hb or units/gm Hb and calculated by calibration curve.

3.3.3.8 SOD (Superoxide Dismutase) activity assay (Mishra H.P. & Fridovich, 1972).

Reagents required are Epinephrine or adrenaline (1.8mM), Sodium carbonate buffer (0.3M, pH=10.2) (Na_2CO_3 + $NaHCO_3$), 0.6 mM of EDTA (ethylene di-amine tetra

acetic acid). Sample prepration included adding 0.1ml RBC Hemolysate to 0.9ml triple distilled water. In a test tube 0.5ml diluted sample was taken. To it, 0.5ml sodium carbonate buffer (0.3M, pH 10.2), 0.5ml EDTA (0.6mM), 1.0ml triple Distilled H_2O and 0.5ml epinephrine (1.8mM) was added. Increase in absorbance at 480nm was measured in every 30 seconds till 2.5 minutes.

For blank, in a test tube 0.5ml buffer (Sodium carbonate) was taken to it 0.5ml sodium carbonate buffer (0.3M, pH 10.2), 0.5ml EDTA (0.6mM), 1.0ml triple Distilled H_2O and 0.5ml epinephrine (1.8mM) was added. Instead of sample, 0.5 ml buffer (Sodium carbonate) was added. Increase in absorbance at 480nm was measured in every 30 seconds till 2.5 minutes.

Calculations-

Specific activity of enzyme (SOD) = $\frac{\text{Units per ml enzyme}}{\text{Hb gm/dl}}$

Unit per ml enzyme = 50% inhibition/0.1 X 50

Percentage inhibition (%) = $\frac{X \times 100}{A}$

50% inhibition = 100% inhibition/2

Where,

x = O.D. change in experimental reaction–O.D. change in control/blank reaction

A = O.D. change in experimental reaction

3.3.3.9 Glutathione Peroxidase (GPx) Activity assay (Hafeman D.G. method, 1974)

Reagents required/prepared:-

1. Metaphosphoric acid solution (1.67 gm Metaphosphoric acid + 0.2 gm EDTA + 30 gm NaCl + 100 ml triple distil water).

2. DTNB Reagent (40mg of di-thiobis nitro benzoic acid in 100ml of 1% w/v tri-sodium citrate solution).

3. 0.4M Sodium phosphate buffer:- pH = 7.0 (Na_2HPO_4 + NaH_2PO_4)

4. 0.01M Sodium Azide (NaN_3)

5. 1.25mM Hydrogen peroxide (H_2O_2)

6. 2mM GSH (glutathione reduced) as standard.

Procedure: -

Preparation of sample for Test:-

0.3ml of haemolysate was taken in a test tube, 1ml of 0.4M Sodium phosphate buffer (pH=7), 0.5ml of 0.01M NaN_3 and 1ml of 2 mM of GSH was added to it.

Preparation of standard: -

1.0ml of 2mMol of GSH was taken in a test tube and 1ml of 0.40M Sodium phosphate buffer (pH) was added to it. 0.5ml of 0.01M NaN_3 (Sodium azide) was mixed.

Preparation of Blank: -

1ml of water or 0.15M KCl was taken in a test tube, 1ml of 0.40M Sodium phosphate buffer and 0.5ml of 0.01M NaN_3 was added to it.

Procedure for the test: -

Volume of all three test tubes was made up to 4ml and was kept for 5 minutes, 1ml of 1.25mM H_2O_2 (pre-warmed to 37°C) was added to each of the three tubes i.e. test, standard & blank, after 3 minutes,1ml of aliquot from all three test tubes was taken. 4ml of metaphosphoric precipitating solution was added in each test tube and it was centrifuged to collect supernatant. 2ml of supernatant from each test tube was taken and 2ml of 0.4M phosphate solution and 1ml of DTNB to each test tube was added. O.D. at 412nm within 2 minutes was read.

Calculations:

Specific activity of enzyme = (O.D. of test/ O.D. of standard) * Units of enzyme per ml conc. Of Hb in gm %(percent).

3.3.3.10 Glutathione Reductase activity assay (Bergmeyer H.U.,1963)

Reagents required are Phosphate buffer (0.1M; pH 7.5) (Na_2HPO_4 + KH_2PO_4), 0.2M GSSG (oxidized glutathione), NADPH (0.12mM).

Sample prepration, 1ml RBC hemolysate + 0.9ml T.D.W. i.e. 1:10 dilution was taken. In a test tube Phosphate buffer (2100µl), GSSG (300µl), Diluted haemolysate (300µl), NADPH (300µl) were added, then O.D. at 340nm in decreasing order on time scale 7 times after 30 seconds was taken.

Calculations were done as follows

$$\text{Specific activity of GR} = \frac{\text{OD Change Per Minute}}{6.3 \times 10^3} \times \frac{\text{ml of reaction mixtre}}{\text{ml of sample volume}} \times 10^6$$

3.3.3.11 ALDOSE REDUCTASE (AR) activities in RBC hemolysate (Nishimura C, Hamada Y, 1994).

Reagents required/prepared: -

1. Phosphate buffer (0.1M; pH 6.2) (Na_2HPO_4 + KH_2PO_4).
2. 0.4 m mol lithium sulphate.
3. 5 µmol 2-mercapto ethanol.
4. 10 µmol DL-glyceraldehyde.
5. 0.1 µmol NADPH.

Preparation of sample: -

0.1ml RBC hemolysate + 0.9ml T.D.W. i.e. 1:10 dilution was taken.

Procedure: -

In a test tube these reagents were added: -

1 Phosphate buffer 2100µl

2 2-mercapto ethanol 200µl

1. Haemolysate 200µl

2. NADPH 100µl
3. DL-glyceraldehyde 200µl
4. Lithium sulphate 200µl

And O.D. at 340nm in decreasing order on time scale 7 times after 30 seconds was taken.

Calculations:

Specific activity of AR = $\frac{\text{O.D. change per minute}}{6.3 \times 10^3} \times \frac{\text{ml of reaction mixture}}{\text{ml of sample volume}} \times 10^6$

Specific activity of AR = $\frac{\text{O.D. change per minute}}{6.3} \times \frac{\text{ml of reaction mixture}}{\text{ml of sample volume / Hb gm dl}^{-1}} \times 10^3$

3.3.3.12 Glucose-6-Phosphate Dehydrogenase (G-6-PD) Activity in whole blood by G-SIX kit method.

Reagents required/prepared:-

1. L1: G-6-PDH Reagent
2. L2: Starter Reagent

Procedure for the test:

In a test tube these reagents were added:

G-6-PD Working Reagent 1ml

Whole Blood 0.01ml

Mix well & incubate for 5-10 min at 37^0C and added

Starter Reagent 2.0 ml

Incubate at 37^0C for 5 min and taken O.D. at 340nm and read the initial absorbance every 1, 2 and 3 min.

Calculation

G-6-PDH Activity (U/g Hb) = ΔA x 4778/ Hb (g/dl)

A/min = A2-A1/ 5

3.3.3.13 Trace metal Analysis

Copper

It was estimated by colorimetric method using kit from Coral clinical systems (Goa, India).

Principle

Copper, released from ceruloplasmin in an acidic medium, reacts with Di-Br-PAESA to form a coloured complex. Intensity of the complex formed is directly proportional to the amount of Copper present in the sample.

Reagents (75 ml Kit)

L1: Buffer Reagent - (37.5 ml)

L2: Colour reagent - (37.5ml)

S: Copper standard (200µg/dl) - (2ml)

Addition sequence	**Blank (ml)**	**Standard (ml)**	**Test (ml)**
Buffer Reagent (L1)	0.5	0.5	0.5
Colour Reagent (L2)	0.5	0.5	0.5
Distilled water	0.05	-	-
Copper Standard (S)	-	0.05	-
Sample (serum)	-	-	0.05

Reaction mixture was mixed well and incubated at room temperature (25^{o}C) for 10 min. The absorbance of the standard (Abs.S) and test sample (Abs.T) was read against blank within 30 min. at 578nm.

Calculations

Copper (µg/dl) = (Abs.T/Abs.S)*200

Normal range(Serum)

Male: 80- 140 µg/ dl

Females: 80- 155 µg/dl

Zinc

It was estimated by colorimetric method using kit from Coral clinical systems (Goa, India).

Principle

Zinc in an alkaline medium reacts with Nitro-PAPS to form a purple coloured complex. Intensity of the complex formed is directly proportional to the amount of Zinc present in the sample. Normal range in serum is 60-120 µg/dl.

$$\text{Zinc} + \text{Nitro-PAPS} \xrightarrow[\text{Alkaline medium}]{} \text{Purple colour complex}$$

Reagents (75 ml Kit)

L1: Buffer Reagent - (60 ml)

L2: Colour reagent - (15ml)

S: Zinc standard (200µg/dl) - (2ml)

Working reagent- 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2)

Addition sequence	Blank (ml)	Standard (ml)	Test (ml)
Working Reagent	1.0	1.0	1.0
Distilled water	0.05	-	-
Zinc Standard (S)	-	0.05	-
Sample (serum)	-	-	0.05

Reaction mixture was mixed well and incubated at room temperature (25^{o}C) for 5 min. The absorbance of the standard (Abs.S) and test sample (Abs.T) was read against blank within 20 min. at 578nm.

Calculations

Zinc (µg/dl) = (Abs.T/Abs.S)*200

Normal Range (serum): 60-120 µg/dl

Iron and TIBC (Total Iron Binding Capacity)

It was estimated by ferrozine method using kit from Coral clinical systems (Goa, India).

Principle

Iron bound to transferrin, is released in an acidic medium and the ferric ions are reduced to ferrous ions. The Fe (II) ions react with ferrozine to form a violet coloured complex. Intensity of the complex formed is directly proportional to the amount of iron present in the sample. For TIBC, the serum is treated with excess of Fe (II) to saturate the iron binding sites on transferring. The excess Fe (II) is adsorbed and precipitated and the iron content in the supernatant is measured to give the TIBC.

Iron Reagents (75 ml kit)

L1: Iron Buffer Reagent (75 ml)

L2: Iron Colour Reagent (4 ml)

S: Iron Standard (100µg/dl) (2 ml)

TIBC Reagents

L1: TIBC Saturating Reagent (20 ml)

L2: TIBC Precipitating Reagent (20 ml)

Iron Assay

Addition Sequence	**Blank (ml)**	**Standard (ml)**	**Sample Blank (ml)**	**Test (ml)**
L1	1.0	1.0	1.05	1.0
Distilled water	0.2	-	-	-
Standard	-	0.2	-	-
Sample	-	-	0.2	0.2
L2	0.05	0.05	-	0.05

The reaction mixture was mixed well and incubated at room temperature (25^{o}C) for 10 min. The absorbances of Blank (Abs.B), Standard (Abs.S), Sample Blank (Abs.SB) and Test (Abs.T) were measured against distilled water at 578nm.

Calculations

Iron (µg/dl) = [(AbsT – (Abs SB + Abs B))/Abs S – Abs B]*100

TIBC Assay

Serum +TIBC Saturating Reagent (L1)	0.5ml + 1.0 ml
Mixed well and allowed to stand for 10 min. at 25^{o}C	
TIBC Precipitating Reagent (L2) was added	Approx. 50mg

Reaction mixture was centrifuged at 2500-3000 rpm for 10 min. to obtain a clear supernatant. The iron content was determined in the supernatant as mentioned above in iron assay.

TIBC (µg/dl) = [(AbsT – (Abs SB + Abs B))/Abs S – Abs B]*300

3.4 Genetic polymorphism study

3.4.1 DNA Isolation and storage

DNA isolation from blood tissue was done by using standard phenol-chloroform protocol (Somasundaram et al., 2002). 600 µL blood was taken, washed in High T.E. buffer (1M Tris base, 0.5 M EDTA) and centrifuged at 20 °C at 8500 rpm for 10 min. The supernatant was decanted and the pellet was homogenized with SET buffer (pH 8) twice and centrifuged at 20 °C at 8500 rpm for 10 minutes. Supernatant was discarded, and 1 mL TEN buffer (pH 8), 600 µL 10 % SDS and 5 µL of 20mg/mL of protenaise K was added and was left for overnight incubation at37 °C. Tris saturated phenol: chloroform: isoamyl alcohol: 25:24:1 was added to the tube and the latter centrifuged at 8500 rpm at 20 °C for 10 minutes. The supernatant was removed and the pellet resuspended in sodium acetate and chilled absolute alcohol and incubated for 2 hours. Centrifugation at 8500 rpm at 4 °C for 10 minutes was carried out. The DNA pellet was then washed with 70% alcohol and resuspended in TE buffer (pH 8).

The quantity and purity of DNA was checked by measuring optical density (OD) at 260 nm. The ratio of absorbance at 260 and 280 nm of DNA should be around 1.7-1.9 for a good quality DNA. The concentration of DNA was obtained by the following formulae.

OD_{260nm}*Dilution factor/20 =mg/ml

The quality and purity was confirmed by 1% agarose gel electrophoresis in 1XTBE buffer. DNA was stored at 4 degree Centigrade and for longer duration at -20 degree centigrade.

3.4.2 PCR Amplification

Polymerase chain reaction is a powerful method for amplifying specific regions of DNA. Different PCR based techniques are now used for genetic studies and genotyping

of single nucleotide polymorphisms. The two PCR based techniques used for genotyping in this study are primer amplification refractory mutation system PCR (ARMS PCR) and restriction fragment length polymorphism PCR (RFLP PCR). All reagents used for PCR amplification (dNTPs, Taq polymerase, PCR buffer and primers) were obtained from Bangalore Genei (India), Genetix Biotech Asia Pvt Ltd and Fermentas (Lithuania). Primers for each of the SNP are listed in table. Briefly each reaction contained 100-150 ng DNA, primers, $MgCl_2$, PCR buffer, dNTPs and Taq DNA polymerase. All PCR reactions then underwent various cycling conditions for amplification, which were carried out in thermal cycler. Reactions involving initial denaturation followed by varying cycles of denaturation, annealing and extension and a final extension step in some.

3.4.2.1 Amplification Refractory Mutation System (ARMS PCR)

ARMS PCR is a relatively economical method for single nucleotide polymorphism (SNP) genotyping involving a single PCR reaction followed by agarose gel electrophoresis. It is an allele specific PCR genotyping technique which makes use of two sets of primers designated outer primers and inner primers. The outer primers are constant whereas the inner primers are allele specific. A deliberate mismatch is included in the -3 position of the inner allele specific primers for increasing specificity. This technique was used to genotype A1298C and T869C SNP in our study population.

The PCR analysis of MTHFR as well as TGF ß gene polymorphism was carried out in a total volume of 50 µl, containing 100 ng of genomic DNA; 10 pmol of each primer; 1X Taq polymerase buffer, dNTPs (200µM each) and 1.5 units of Taq DNA polymerase. The PCR product was electrophoresced on 2% agarose gels for 45 min after which the gels were visualized on a Gel Doc system (BioRad,USA) to detect the SNP.

3.4.2.2 Restriction Fragment Length Polymorphism (RFLP) PCR

RFLP PCR utilizes the specificity of restriction enzymes, for SNP genotyping. SNPs present in restriction enzyme sites can be genotyped using this technique. For RFLP PCR the fragment of interest wasamplified from the DNA samples and was then restricted overnight with the specificrestriction enzyme followed by agarose gel electrophoresis for genotyping. Genotyping of the PTPN22-1858C/T SNP was performed by PCR-restriction fragment length polymorphism.

In a PCR tube, GoTaq® Green Master Mix, 2X was taken 12.5µl and added the primer (10 pmole) forward and reverse 0.5 µl respectively. 2 µl genomic DNA (100 ng) was taken. 0.3 µl taq polymerase was added. Nuclease free water was added upto the final volume gain 25 µl. In PCR reactions, First step was denaturation at 94°C for 5 min. followed by denaturation, done at 94°C for 55 sec. In the third step Annealing was done at temperature (depends on the primer sequence) for 45 sec. Fourth step was the extension of the reaction at the temperature of 72°C for 30 sec. Repeated the step 2 to 4 for 30 cycles and final extension was done at 72°C for 5 min. After PCR, DNA fragments were digested with RsaI restriction enzyme (Fermentas, USA) and incubated at 37oC overnight. Samples were visualized on 2% agarose gels and ethidium bromide staining was used to detect amplified fragments. Intact PCR products before digestion provided a band at 275 bp.

Primers

Both the reverse and forward primers were obtained from Invitrogen Life Technologies or Amersham Pharmacia Biotech in a lyophilised form and reconstituted to 100 µM stock solution in deionised water. A 1:10 dilution of the stock solution was made in deionised water and the resultant 10 µM solution was aliquoted in 50 µl volumes and stored at -20°C before use. The 10 µM aliquots of primers were preferably used within six months. Thawing and re-freezing cycles were kept to a minimum to reduce

denaturation of primers. List of forward and reverse primers for the genes studied is given in Table 3.1.

Table 3.1 - Primer sequences used for amplification of different genes

Name of gene	Primer sequence
TGFß 1	Sense 5'- TCC GTG GGA TAC TGA GAC AC-3' C-Antisense 5'-GCA GCG GTA GCA GCA GCG-3' T-Antisense 5'-AGC AGC GGT AGC AGCAGC A-3,
MTHFR	Sense 5'- CCA CTC CAG CAT CAC TCA CT-3' A-Antisense 5'-GGA GGA GCT GAC CAG TGA ATA-3' C- Antisense 5'-GGA GGA GCT GAC CAG TGA ATC-3'
PTPN22	Sense 5'-GATAATGTTGCTTCAACGGAATTT-3' Antisense 5'-CCATCCCACACTTTATTTTATACT-3'

3.5 Metabolomics study by NMR

3.5.1 NMR Metabolomics-Sample Collection and Preparation

In each case, 3.0 ml of blood sample was drawn and processed to extract the serum as per the established protocol. Thecollected serum was transferred into a sterile 1.5 ml microcentrifuge tube (MCT) and stored at -80^{o}C immediately after the processing until the NMR experiments were performed. The serum samples of type 2 diabetic patients without complications (DB) and type 2 patients with complication (DC) along with age and sex matched normal healthy controls(NC) were used in this study to profile the metabolic differences.

All the serum samples were thawed and centrifuged at 10,000 rpm for 5 minutes to remove precipitates just before acquiring the NMR data. Serum were prepared as followed: 250 µl of serum was collected in a sterile centrifuge tube, final volume adjusted by adding 250 µl of 0.9% saline sodium phosphate buffer of strength 20 mM and pH 7.4 prepared in D_2O. A total of 400 µl of sample was used in 5 mm NMR tubes with a coaxial insert containing known concentration of TSP (0.1mM prepared in D_2O) to provide lock for NMR experiments, and as an external standard reference to aid

chemical shift referencing for metabolite quantification and assignment. [D_2O (Deuterium oxide; as a co-solvent and to provide a deuterium field/frequency lock) and TSP (sodium salt of trimethylsilylpropionic acid-d4] used for NMR experiments were purchased from Sigma-Aldrich (Rhode Island, USA).

3.5.2 NMR Measurements:

All NMR spectra were recorded at 298 K on BrukerBiospinAvance-III 800 MHz NMR spectrometer operating at proton frequency of 800.21 MHz, equipped with CryoProbe and an actively shielded gradient unit with a maximum gradient strength output of 53 G/cm. The raw NMR data were processed in Topspin-2.1 (Bruker NMR data Processing Software). For each serum sample, transverse relaxation-edited CPMG (Carr–Purcell–Meiboom–Gill) spectra 1D ^1H NMR spectra were recorded using the standard Bruker's pulse program library sequence(cpmgpr1d) with pre-saturation of the water peak through irradiating it continuously during the recycle delay (RD) of 5 sec. Each spectrum consisted of the accumulation of 128 scans and lasted for approximately 15 minutes. To remove broad signals from triglycerides, proteins, cholesterols and phospholipids, a total spin–spin relaxation time of 60 ms (n=300 and 2□=200□s) was applied. Each FID (free induction decay) was zero filled and Fourier-transformed to 64 K data points following manual phase and baseline-correction using Bruker NMR data Processing Software Topspin-v2.1. A line broadening factor of 0.3 Hz and a sine–bell apodisation function was applied to FIDs before Fourier Transformation. After FT, the chemical shifts were referenced internally to methyl peak of L-lactate (at δ=1.33 ppm). All recorded spectra were, visually inspected for acceptability and subjected to multivariate statistical analysis to identify the altered metabolic patterns.

3.5.3 Identification of Metabolite Peaks:

Chemical shifts were identified and assigned as far as possible, by comparing them with the chemical shifts available with the open access software program MetaboMiner25 with tolerances of 0.05 ppm (^{1}H) and 0.1 ppm (^{13}C). The metabolite peaks were identified if there was only one candidate in the database within the specified tolerances for an observed peak and its correlated shifts. The metabolite peaks in one-dimensional ^{1}H CPMG NMR spectra were identified and assigned as far as possible, by comparing them with the chemical shifts available with the software Chenomx (NMR Suite, v8.1, Chenomx Inc., Edmonton, Canada). The assigned resonances of the metabolite peaks were validated using: (a) previously reported NMR assignments of metabolites, data obtained from BMRB database (Biological Magnetic Resonance Data Bank) and HMDB (The Human Metabolome Database) and (b) Assigned resonances in two-dimensional spectra. For unambiguous assignment of various peaks in these spectra, two-dimensional (2D) ^{1}H–^{1}H TOCSY (Total Correlation Spectroscopy) and ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum correlation) NMR spectra were also acquired at 298 K for some of the serum samples using the parameters as described previously. The assignments of peaks from lipid moieties were obtained based on previous literature reports.

3.5.4 Statistical Analysis

The ^{1}H NMR spectra of all the serum samples were manually phase adjusted and baseline corrected after referencing to the alanine resonance at δ(1.46) ppm. The CPMG □(8.5-0.7) ppm spectra were binned and automatically integrated using AMIX package (Version 3.8.7, Bruker, BioSpin), to reduce the complexity of the NMR data and facilitate pattern recognition. The region distorted due to water suppression δ(5.1-4.7) ppm, were excluded from the CPMG data set. Finally, the selected regions were

reduced to spectral bins of δ (0.01) ppm. The resultant CPMG, data sets were finally used for univariate and multivariate analysis in statistical analysis module of *MetaboAnalyst*, an open access web-based tool for metabolomics studies.

In the present article, we chose RF to select differentiating biomarkers between the different cohorts i.e diabetes (DB), diabetes related complication (DC) and control (NC). Prior to multivariate statistical analysis the binned dataset were pareto scaled, which is the recommended method for untargeted metabolomics studies. Subsequently the normalized dataset was subjected to supervised classification methods— Random forest (RF). RF is a classification and regression technique which involves constructing a multitude of trees in the training phase. More precisely saying, it is an ensemble method of trees developed from a training dataset and validated internally to achieve an accurate prediction of the target variable from the predictors for the purpose of future observations. RF will create multiple classification and regression trees (CART) based on the bootstrap sample from the original training data. It also randomly searches the feature to determine the splitting point for growing a tree. In addition, the RF does not over fit as the number of trees increases but it will produce a restrictive value on the generalization error. Random Forests is best suited for the analysis of complex data structures embedded in small to moderate data sets. Its strengths are spotting outliers and anomalies in data, displaying proximity clusters, predicting future outcomes, identifying important predictors, discovering data patterns, replacing missing values with imputations, and providing insightful graphics. The important variable/features were ranked by their contributions to classification accuracy i.e. Mean Decrease Accuracy (MDA). Furthermore, unpaired t-test was applied to assess the significance of change in the metabolic profile and p-value < 0.05 was used as the criterion for statistical significance. Receiver operating characteristic (ROC) analysis was also

carried out to verify the robustness of discriminatory metabolites, which are generated by plotting the false positive rate against true positive rate and area under the curve (AUC) values were calculated as an indication of the prediction accuracy. ANOVA was performed for boxplot representation to visualize the comparativevariation in the levels of significantly altered metabolites in different cohorts (DB, DC and NC) identified in the multivariate analysis.

CHAPTER 4:

RESULTS

RESULTS

4.1 Anthropometric features

All the 120 diabetic patients screened had complete records of their clinical profile. Table 4.1 comprises of the anthropometric features of the subjects. The mean value of diabetes duration was 7.07±0.5 years.

Table 4.1- Anthropometric features of controls and type 2 diabetic subjects

Features	Controls (n=120)	Diabetics (n=120)
BMI (Kg/m^2)	25.36±0.26	27.27±0.24*
Disease duration (years)	-	7.07±0.5
SBP (mmHg)	125.79±0.84	143.24±1.45*
DBP (mmHg)	81.34±0.48	87.73±1.09*

n=no. of subjects, BMI= Body Mass Index, SBP=Systolic Blood Pressure, DBP=Diastolic Blood Pressure, values are represented as mean±SEM (standard error of mean), * statistically significant as compared to controls.

BMI of the diabetic patients was significantly increased (27.27±0.24) Kg/m^2 as compared to controls (25.36±0.26) kg/m^2 ($p<0.001$). When the values were compared gender wise, both male and female patients showed significant increase in BMI values as compared to their respective controls (fig) ($p<0.001$). The mean BMI of diabetic females (28.3±0.51) Kg/m^2 was the highest among all the subjects.

The systolic blood pressure and diastolic blood pressure values of patients were found to be significantly increased in patients as compared to controls (table 4.1) ($p<0.001$). Similar trend was observed when the comparison was made on the basis of gender (fig) ($p<0.001$).

4.2 Socio-economic and demographic features of T2DM patients and controls

Table 4.2 - Socio-economic and demographic features of subjects*

Parameter	Controls	Diabetics	P value
Age	50.76±1.0	53.13±0.91	-
Gender M/F	69/51	83/37	-
Economic status (a)LIG	24(20%)	32(26.66%)	χ^2=3.33, df=2, p=0.1888
(b)MIG	50(41.66%)	46(38.33%)	not significant
(c)HIG	46(38.33%)	42(35%)	
Marital status(a)single	35(29.16%)	17(14.16%)	χ^2=13.07, df=2, p<.0015
(b)Married	80(66.66%)	97(80.83%)	statistically significant
(c) Widowed	5(4.16%)	6(5%)	
Education(a) Junior	4(3.33%)	18(15%)	χ^2=19.27,df=3,p=.0002
(b) Intermediate	31(25.83%)	29(24.16%)	statistically significant
(c) Graduate	47(39.16%)	49(40.83%)	
(d) University	38(31.66%)	24(20%)	
Occupation (a)service	52(43.33%)	61(50.83%)	χ^2=28.89,df=3,p<.0001
(b) business	26(21.66%)	32(26.66%)	statistically significant
(c) unemployed	30(25%)	6(5%)	
(d) others	12(10%)	21(17.5%)	
Smoking (a)No	70(58.33%)	98(81.66%)	χ^2=15.55,df=2,p<.0001
(b)Yes	50(41.66%)	22(18.33%)	statistically significant
Exercise (a) Mild	87(72.5%)	101(84.16%)	χ^2=9.44,df-2,p=0.0089
(b) Moderate	17(14.16%)	13(10.83%)	statistically significant
(c) Vigorous	16(13.33%)	6(5%)	
Drinking (a) No	93(77.5%)	95(79.16%)	χ^2=0.09,df=1,p=0.75
(b)Yes	27(22.5%)	25(20.83%)	not significant
Cooking medium (a) Dalda	11(9.16%)	8(6.66%)	χ^2=9.67,df=2,p=.0079
(b) refined oil	56(46.66%)	73(60.83%)	statistically significant
(c) mustard oil	53(44.16%)	39(32.5%)	
Consumption of junk food (a)rarely	5(4.16%)	4(3.33%)	χ^2=7.527,df=2,p=0.0232
(b) sometimes	57(47.5%)	72(60%)	statistically significant
(c) frequently	58(48.33%)	44(36.66%)	

**values expressed in frequency (percentage)*

4.2.1 Gender and age distribution

Gender distribution in controls was 57.5% males (n=69) and 42.5% females (n=51). The age of healthy controls ranged from 30 to 73 years with a mean age of 50.76±1.0 years. Of these 11.6% males (n=8) and 5.88% females (n=3) were in <35 age group, 18.84% males (n=13) and 21.57% females (n=11) were in 36-45 age group and 47.8% males

(n=33) and 56.86% females (n=29) were in 46-60 age group and 21.74% males (n=15) and 15.68% females (n=8) belonged to >60 age group.

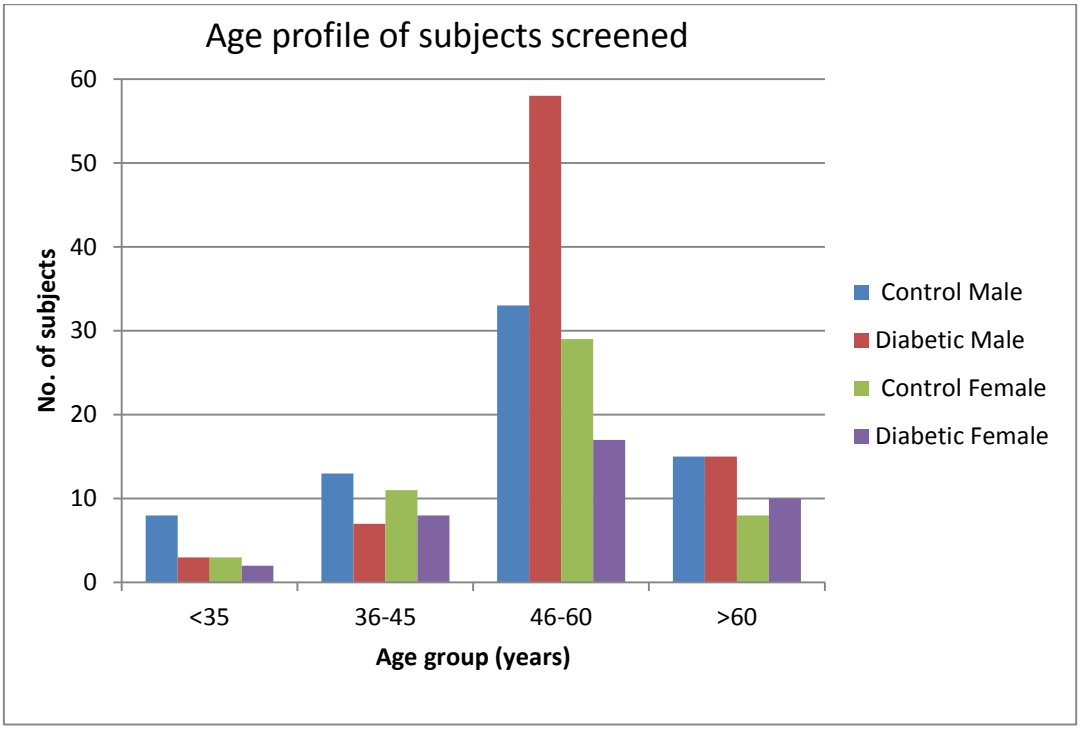


Figure 4.1: Age profile distribution of subjects according to gender

Gender distribution in diabetic patients was 69.16% males (n=83) and 30.83% females (n=37). The age of diabetic patients ranged from 30 to 75 years with a mean age of 53.13±0.91 years. Of these 3.61% males (n=3) and 5.4% females (n=2) were in <35 age group, 8.43% males (n=7) and 21.62% females (n=8) were in 36-45 age group and 69.88% males (n=58) and 45.94% females (n=17) were in 46-60 age group and 18.1% males (n=15) and 27.03% females (n=10) belonged to >60 age group.

4.2.2 Economic status

Diabetic subjects were present in all the three economic strata with highest (38.33%) in MIG followed by HIG (35%) and LIG (26.66%). Similar trend was observed in controls However it was not statistically significant.

4.2.3 Marital status

Majority of the diabetic subjects were married (80.83%) while the rest were single (14.16%) and widowed (5%). Similar trend was seen among the controls with 66.66% of the controls being married, followed by 29.16% of them being single while 4.16% of the controls were widowed. Chi square analysis (χ^2=13.07, degreeof freedom=2) showed that the comparison was highly significant with a p value <0.0015.

4.2.4 Education

The education profile pointed out that about 40.83% patients were graduates while slightly less than a quarter of them (24.16%) were intermediate qualified, one-fifth (20%) of the patients held university degrees and 15% were junior high school pass outs. Majority of the controls were graduates (39.16%), while 31.66% were university degree holders, followed by Intermediate pass outs (25.83%) and junior high school qualified (3.33%). The chi square analysis (χ^2 =19.275, degree of freedom=3) that the comparison was highly significant with a p value of 0.0002

4.2.5 Occupation

Servicemen appeared to be the frontrunners in the occupation section (50.83% diabetics) followed by business men (26.66%), unemployed (5%) and others (17.5%). In controls, 43.33% were servicemen, followed by 25% of them being unemployed, 21.66% being businessmen and 10% belonging to the category of others. The chi square analysis (χ^2 =28.892, degree of freedom=3) showed that the comparison was highly significant with a p value < 0.0001.

4.2.6 Smoking

18.33% (n=22) of the patients replied yes when asked about smoking as compared to 41.66% (n=50) of the controls. 81.66% (n=98) of the diabetic patients denied being a smoker in comparison to 58.33% (n=70) of the controls. The chi square analysis (χ^2

=15.55, degree of freedom=1) showed that the comparison was highly significant with a p value < 0.0001.

4.2.7 Drinking

20.83% (n=25) patients replied yes when asked about alcohol consumption as compared to 22.5% (n=27) of the controls while 79.16% (n=95) of the patients denied alcohol intake as compared to 77.5% (n=93) of the controls.

4.2.8 Physical activity

Mild physical activity was the most prevalent form of exercise among the patients (84.16%) as well as controls (72.5%). It was followed by moderate physical activity (10.83% patients vs. 14.16% controls) and vigorous physical activity (5% patients vs. 13.33% controls).

4.2.9 Cooking medium

Refined oil was the chosen cooking medium among the study subjects (60.83% patients vs. 46.66% controls) followed by Dalda ghee (6.66% patients vs. 9.16% controls) and mustard oil (32.5% patients vs. 44.16% controls). The chi square analysis (χ^2 =9.677, degree of freedom=2) showed that the comparison was highly significant with a p value 0.0079.

4.2.10 Consumption of junk food

60% of the patients admitted to consuming junk food sometimes compared to 47.5% of the controls. 36.66% of the patients enjoyed it frequently as compared to 48.33% of controls. It was reported to be rarely consumed by 3.33% of the patients as against 4.16% of the controls. The chi square analysis (χ^2 =7.527, degree of freedom=2) showed that the comparison was significant with a p value 0.023.

4.2.11 Distribution of subjects as per family history

Distribution of subjects according to family history has been displayed in Table 4.3 and Figure 4.2. 67.5% diabetics had a family history indicating the strong genetic component of the disease. 9.87% of the diabetic males showed positive family history, while 62.16% of the diabetic females exhibited positive family history. However, 14.16% controls also showed family history suggesting their susceptibility for development of the disease. 15.94% control males were found to have positive family history, while 11.76% of the control females exhibited it.

Table 4.3: Distribution of subjects (controls and diabetics) according to family history of T2DM

Family history	Controls	Diabetic	Total
Yes	17 (14.16%)	81 (67.5%)	98
No	103 (85.83%)	39 (32.5%)	142
Total	120	120	240

χ^2=155.594 df=1, p<0.0001

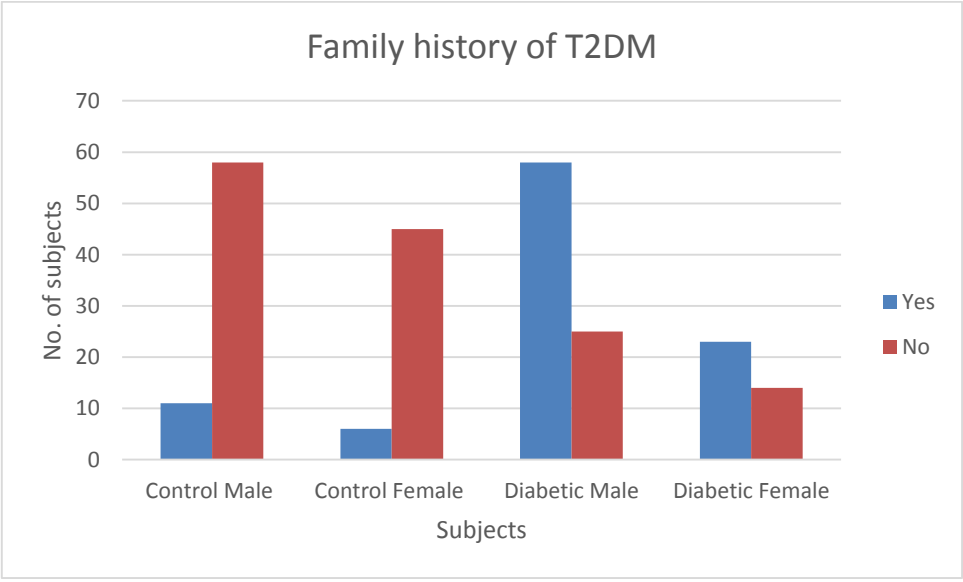


Figure 4.2: Distribution of subjects according to family history

4.3 Biochemical Studies

4.3.1 Estimation of biochemical parameters in T2DM patients and healthy controls

Table 4.4: Variations of biochemical parameters in control and type 2 diabetic subjects

Variable	Controls (n=120)	Diabetics (n=120)
HbA1c (%)	5.57±0.089	7.89±0.083**
Plasma glucose (mg/dl)	108.62±1.44	180.5±5.46**
MDA (µmol/L/mg protein)	0.98±0.02	1.57±0.49**
TC (mg/dl)	183.52±3.29*	234.07±4.10**
TG (mg/dl)	139.14±2.74*	257.36±7.27**
HDL (mg/dl)	40.06±0.61*	33.98±0.52**
LDL (mg/dl)	115.63±3.43*	148.61±4.23**
VLDL (mg/dl)	27.83±0.55*	51.47±1.45**
UA (mg/dl)	3.94±0.08*	5.86±0.16**
Creatinine (mg/dl)	0.995±0.013*	1.068±.024*

HbA1c= Glycated haemoglobin, MDA=Malondialdehyde, TC=Total Cholesterol, TG Triglycerides, HDL= High density Lipoprotein, LDL= Low density Lipoprotein, VLDL=Very Low Density Lipoprotein, UA=Uric Acid. n=no. of subjects, Statistical analysis done by student's t-test (unpaired), Values are mean±SEM (standard error of mean)

* P value<0.05, **p value<0.001.

The levels of various biochemical parameters in T2DM patients and healthy controls are shown in table 4.4 and Figure 4.3. HbA1c which is an indicator of glycaemic control was significantly elevated in patients (7.89±0.083) as compared to controls (5.57±0.089) (p<0.001) (Table 4.4.). MDA which is a marker of lipid peroxidation showed significantly higher values in patients (1.57±0.49) as compared to controls (0.98±0.02) (p<0.001) (Table 4.4.). Significantly higher values of fasting Triglycerides were observed in patients (257.36±7.27) as compared to controls (139.14±2.74)(p<0.001) (Table 4.4). Total Cholesterol was significantly elevated in

patients (234.07±4.1) as compared to controls (183.52±3.29) ($p<0.001$) (Table 4.4). LDL-c was also observed to be significantly elevated in patients (148.61±4.23) as compared to controls (115.63±3.43) ($p<0.001$), whereas HDL-c values were significantly lower in patients (33.98±0.52) as compared to controls (40.06±0.61) ($p<0.001$) (Table 4.4). VLDL-c values were also significantly enhanced in patients (51.47±1.45) as compared to controls (27.83±0.55) ($p<0.001$) (Table 4.4). Significantly higher Uric acid values were observed in patients (5.86±0.16) as compared to controls (3.94±0.08) ($p<0.001$) and Creatinine values were also significantly increased in patients (1.06±0.02) as compared to controls (0.99±0.013) ($p<0.05$) (Table 4.4).

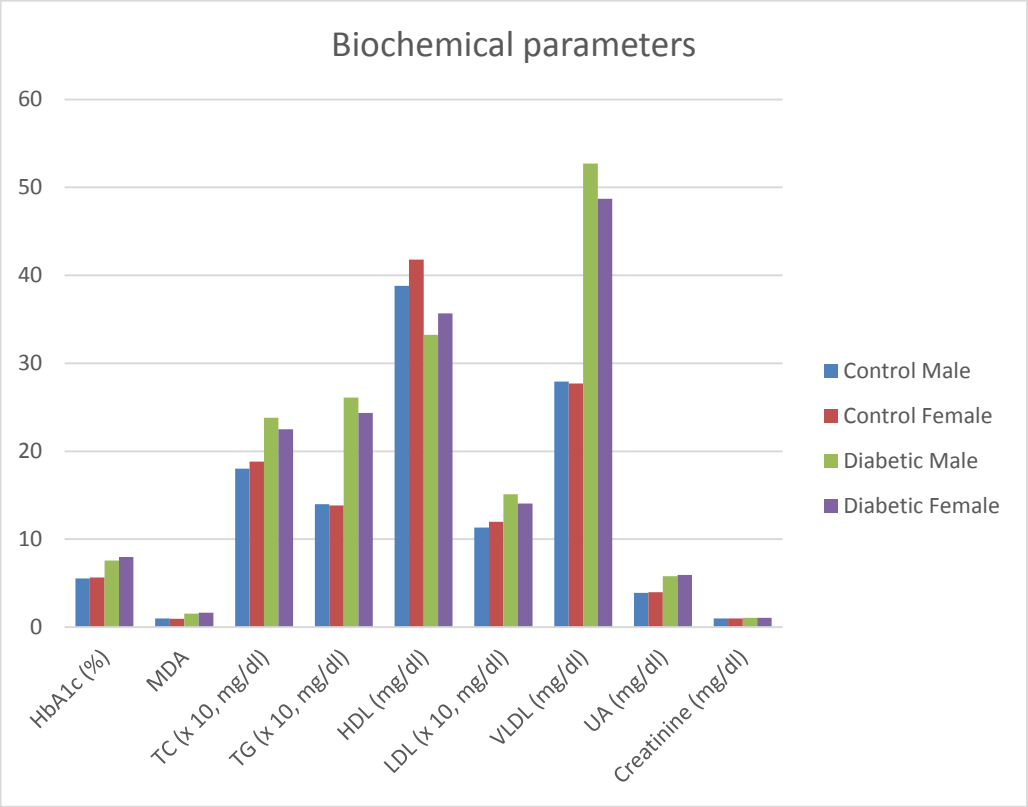


Figure 4.3: Estimation of biochemical parameters

When the comparison was made on the basis of gender, both male and female patients showed significant increase in HbA1c, MDA, TC, TG, LDL, UA and significant decrease in HDL when compared to their respective controls (Figure 4.3).

However, in the case of creatinine, only male patients showed significant increase as compared to male controls (p=0.03), while the difference in values between diabetic females and control females was not statistically significant (p=0.127) (Figure 4.3).

The frequency analysis of various features of the diabetic patients on the basis of gender is shown in Table 4.5.

Table 4.5 Frequency analysis of various features of the diabetic subjects on the basis of gender

Variable	Males (n=83)	Females (n=37)	Total (n=120)
HbA1c> 7	49 (59.03%)	26 (70.27%)	75 (62.5%)
Low HDL-c	74 (89.2%)	26 (70.3%)	100 (83.33%)
High TG	80(96.38%)	35 (94.6%)	115 (95.83%)
High TC	27 (32.53%)	9 (24.32%)	36 (30%)
Hypertension	23 (27.7%)	5 (13.5%)	28 (23.33%)
BMI			
(a) Normal	2 (2.4%)	2 (5.4%)	4 (3.33%)
(b) Overweight	20 (24%)	4(10.8%)	24 (20%)
(c) Obese	61 (73.5%)	31 (83.8%)	92 (76.66%)

High TG ;>150mg/dl, High TC;>250mg/dl, Low HDL-c;<40 for males and <50 for female, Hypertension;systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, BMI (normal;18.5-22.9 Kg/m^2), (overweight;23-24.9 Kg/m^2) and (obese; ≥ 25 Kg/m^2)

Most frequently encountered abnormality among the diabetic patients was high TG values or hypertriglyceridemia (95.83%, n=115), with 96.38% of the males (n=80) and

94.6% of the females (n=35) being affected by it. It was followed by low HDL-c values in 83.33% of the patients (n=100) with 89.2% of the males (n=74) and 70.3% of the females (n=26) being afflicted with it. High TC values or hypercholesterimia was observed in 30% of the patients (n=36). 32.53% of the total males (n=27) and 24.32% of the total females (n=9) shared the derangement. Glycaemic control as evidenced by HbA1c values was found to be suboptimal in 62.5% of the patients. This anomaly prevailed in 59.03% males (n=49) and 70.27% of the females (n=26). Hypertension affected 23.33% of the patients (n=28). Thus, 27.7% of the males (n=23) and 13.5% of the females were found to be affected by it.

BMI profile showed that only 3.33% of the patients (n=4) were in normal weight range. Thus, only 2.4% of the male patients (n=2) and 5.4% of the female patients (n=2) were observed to be normo-weight. 20% of the patients (n=24) were overweight of which male patients were 24% (n=20) and 10.8% of the female patients (n=4) were found to be overweight. Majority of the patients were obese (76.66%, n=92). It was observed that 73.5% of the male patients (n=61) had obesity while 83.8% of the female patients (n=31) suffered from it.

4.3.2 Correlation analysis

The correlation analysis between biochemical parameters of diabetic patients is shown in Table 4.6. Correlation analysis revealed that significant correlation existed between many biochemical parameters. Fasting plasma glucose (FPG) showed significant positive correlation with HbA1c ($r=0.513$, $p<0.0001$), with MDA ($r=0.258$, $p=0.004$), with HDL ($r=0.210$, $p=0.021$) and with LDL ($r=0.235$. $p=0.0096$). HbA1c showed significant positive correlation with MDA ($r=0.232$, $p=0.01$). Total cholesterol (TC) showed significant negative correlation with HDL ($r=-0.272$, $p=0.0026$) and highly significant positive correlation with LDL ($r=0.947$, $p<0.0001$). Triglycerides (TG)

showed highly significant negative correlation with HDL (r=-0.364, p<0.0001). HDL showed highly significant negative correlation with LDL (r=-0.262, p=0.0038).

Table 4.6 Correlation analysis between biochemical parameters

	FPG	HbA1c	MDA	TC	TG	HDL	LDL	UA	Creatinine
FPG	1	r=0.513 p<0.0001**	r=0.258 p=0.004**	r=0.217 p=0.017	r=0.003 p=0.969	r=0.210 p=0.021*	r=0.235 p=0.0096**	r=0.087 p=0.344	r=-0.046 p=0.614
HbA1c		1	r=0.232 p=0.01**	r=0.147 p=0.108	r=0.042 p=0.648	r=-0.153 p=0.094	r=0.175 p=0.054	r=0.073 p=0.425	r=0.049p p=0.59
MDA			1	r=0.001 p=0.99	r=0.103 p=0.261	r=0.063 p=0.491	r=0.027 p=0.769	r=0.028 p=0.76	r=0.011 p=0.90
TC				1	r=0.16 p=0.08	r=-0.272 p=0.0026**	r=0.947 p<0.0001**	r=0.108 p=0.236	R=0.048 P=0.601
TG					1	r=-0.364 p<0.0001**	r=-0.144 p=0.117	r=0.115 p=0.210	r=0.776 p=0.399
HDL						1	r=-0.262 p=0.0038**	r=0.042 p=0.646	r=-0.005 p=0.951
LDL							1	r=0.071 p=0.442	r= 0.021 p=0.821
UA								1	r=0.091 p=0.323
Creatinine									1

r= Pearson correlation coefficient, * Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed),FPG= Fasting plasma glucose, HbA1c= Glycated haemoglobin, MDA=Malondialdehyde, TC=Total Cholesterol, TG Triglycerides, HDL= High density Lipoprotein, LDL= Low density Lipoprotein, VLDL=Very Low Density Lipoprotein, UA=Uric Acid

The correlation analysis of lipid profile with disease duration and age of the diabetic patients is shown in Table 4.7.All the parameters TC, TG, HDL, and LDL showed weak positive correlation with age of the patients but none of them were significant. Similarly, TC, HDL and LDL showed weak positive correlation while TG showed weak negative correlation with disease duration but none of them were found to be significant

Table 4.7 Correlation analysis of lipid profile with disease duration and age of the diabetic subjects

parameter	TC	TG	HDL	LDL
Age	r=0.072 p=0.429^{ns}	r=0.063 p=0.488^{ns}	r=0.001 p=0.99^{ns}	r=0.047 p=0.603^{ns}
Disease duration	r=0.029 p=0.747^{ns}	r=-0.172 p=0.059^{ns}	r=0.013 p=0.885^{ns}	r=0.013 p=0.885^{ns}

r= Pearson correlation coefficient, ns; correlation not significant, TC; Total Cholesterol, TG; Triglycerides, HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein

4.4 Metabolic Syndrome

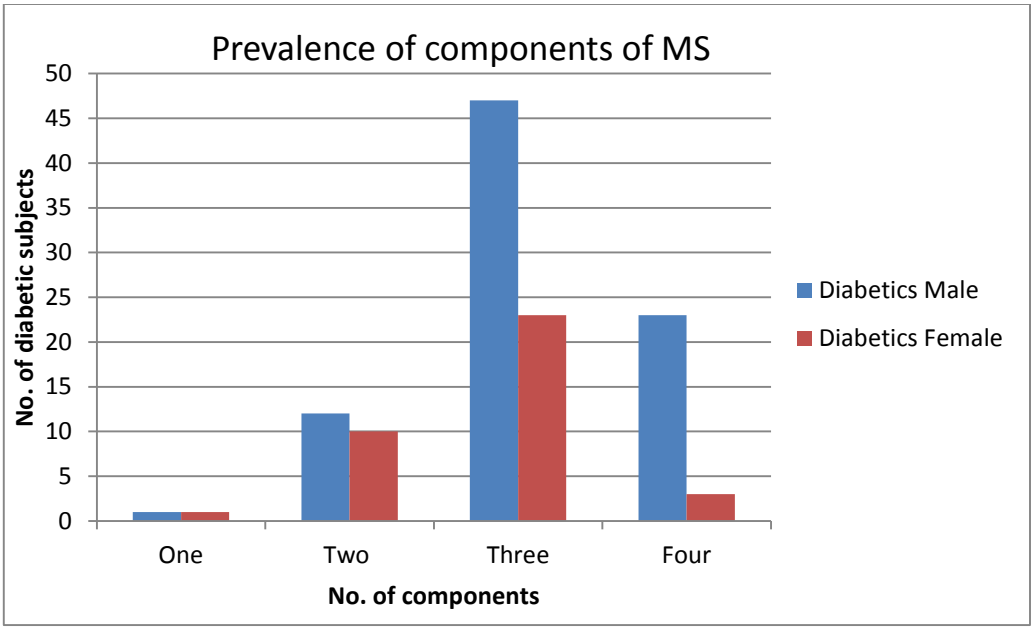


Figure 4.4: prevalence of components of MS according to components

As expected, MS was highly prevalent in diabetic subjects. (58.33 % patients (n=70) displayed three out of five components while 21.66% (n=26) exhibited four components

of the syndrome thus making 80% of the diabetics (n=96) prone to MS. Less than 2% of the diabetics, (1.66%, n=2) suffered from a single component of MS while 18.33% (n=22) displayed two components of the syndrome**.**

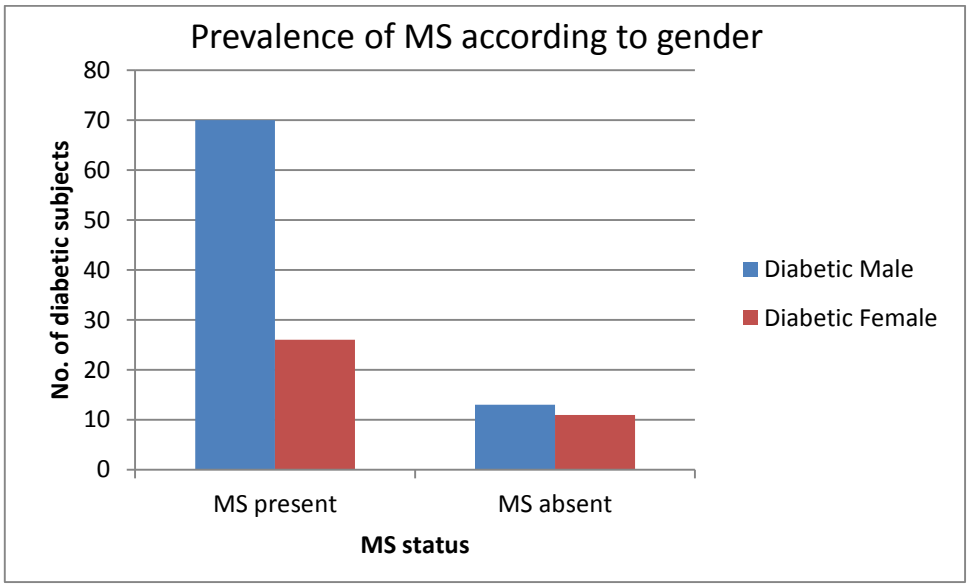


Figure 4.5: prevalence of components of MS according to gender

Out of 96 diabetic subjects diagnosed with MS, 84.34% of the total males (n=70) were afflicted with it while 70.27% of the females had it (n=26).

4.5 Antioxidant enzyme studies

Variations in the activity of antioxidant enzymes in controls and type 2 diabetes patients are shown in Table 4.8. Activity of SOD was found to be significantly increased in patients (1929.38±47.11) as compared to controls (1115.66±50.82) ($p<0.001$). Activity of GPx enzymes in patients (12.15±0.15) was found to be significantly decreased as compared to controls (29.64±0.38) ($p<0.001$). Similarly, activity of catalase enzyme was also significantly diminished in patients (1.45±0.02) as compared to controls (2.76±0.05) ($p<0.001$). Activity of GR enzyme was significantly enhanced in patients (31.71±0.76) as compared to controls (16.14±0.27) ($p<0.001$). Activity of G6PD

witnessed significant decrease in patients (7.0±0.12) as compared to controls (9.75±0.19) ($p<0.001$). On the contrary, activity of AR was observed to be significantly elevated in patients (4.8±0.07) as compared to controls (3.47±0.05) ($p<0.001$).

Table 4.8 Variations in activity of Antioxidant enzymes

Enzyme	Controls (n=120)	Diabetics (n=120)
SOD (U/ml/Hb)	1115.66±50.82	1929.38±47.11*
CAT (U/gHb)	2.76±0.05	1.45±0.02*
GPx (U/gHb)	29.64±0.38	12.15±0.15*
GR (U/gHb)	16.14±0.27	31.71±0.76*
G6PD (U/gHb)	9.75±0.19	7.0±0.12*
AR (U/gHb)	3.47±0.05	4.8±0.07*

SOD;Superoxide Dismutase, CAT;Catalase, GPx; Glutathione Peroxidase; GR; Glutathione Reductase; G6PD; Glucose 6 Phosphate Dehydrogenase; AR; Aldose Reductasen=no. of subjects, Statistical analysis done by student's t-test (unpaired), Values are mean±SEM (standard error of mean) * P value<0.001

Comparison of activity of antioxidant enzymes between controls and diabetic patients on the basis of gender is shown in Figure 4.6. When the enzyme activities were compared on the basis of gender, both male as well as female patients showed significant increase in the activity levels when compared with their respective controls.

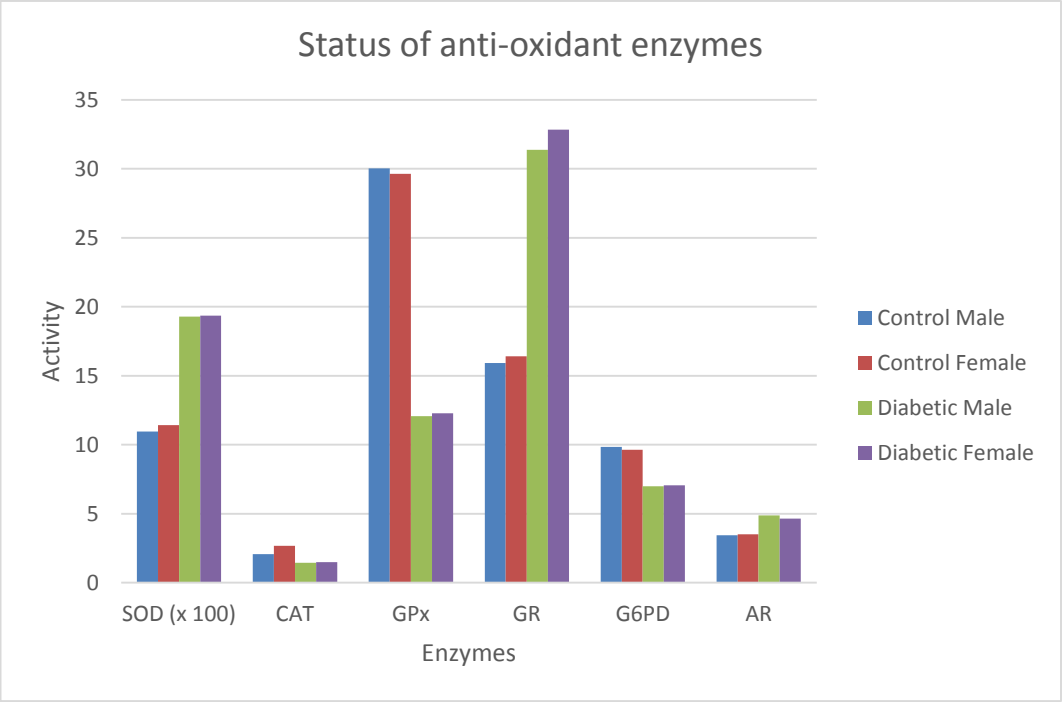


Figure 4.6: Anti-oxidant enzyme activity according to gender

Table 4.9- Correlation analysis between Antioxidant enzymes

	SOD	GPx	GR	CAT	G6PD	AR
SOD	1					
GPx	r=0.044 p=0.632	1				
GR	r=0.744 p=0.419	r=0.182* p=0.045	1			
CAT	r=0.261** p=0.003	r=0.054 p=0.553	r=-0.001 p=0.989	1		
G6PD	r=-o.126 p=0.168	r=0.093 p=0.312	r=-0.057 p=0.581	r=0.084 p=0.359	1	
AR	r=0.023 p=0.795	r=-0.050 p=0.583	r=-0.139 p=0.128	r=0.035 p=0.699	r=0.0151 p=0.869	1

r= Pearson correlation coefficient, * Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed), SOD;Superoxide Dismutase, CAT;Catalase, GPx; Glutathione Peroxidase; GR; Glutathione Reductase; G6PD; Glucose 6 Phosphate Dehydrogenase; AR; Aldose Reductase

The inter-correlation analysis of antioxidant enzymes revealed significant positive correlation between the activities of enzymes GR and GPx (r=0.1828, p=0.045) whereas significant negative correlation between SOD and catalase activities (r=-0.261, p=0.003). Rest all the correlations were weakly positive or negative but were not significant (p>0.05).

4.6 Trace metal analysis

The comparison of levels of copper and zinc in controls and type 2 diabetic patients is shown in Table 4.10.

Table 4.10-Comparative analysis of serum zinc levels among patients with type 2 diabetes mellitus and controls

Trace metal	Controls (n=120)	Diabetics (n=120)
Cu (µg/dl)	120.32±2.04	161.34±1.81**
Zn (µg/dl)	94.73±1.44	90.06±1.36*

Values are mean ± SE, Values in parenthesis represent sample size. Statistical comparison was done among patients of type 2 diabetes mellitus and controls Normal range of serum zinc: 60 to 120 µg/dl. Normal range of serum copper: 80-140 µg/dl in male and 80-155 µg/dl in female. * $p<0.05$, ** $p<0.0001$

As shown in the Table 4.10, the level of copper is significantly increased in diabetic patients (161.34±1.81) as compared to controls (120.32±2.04) ($p<0.0001$). On the contrary, the level of zinc is significantly reduced in diabetic patients (90.06±1.36) as compared to controls (94.73±1.44) ($p=0.0195$).

When the comparison was made on the basis of gender, it was observed that both male and female diabetic patients exhibited significantly elevated levels of copper as compared to their respective controls ($p<0.0001$). However, in the case of zinc, both male and female diabetic patients showed decreased values which were not statistically significant as compared to their respective controls ($p>0.05$) (Figure 4.7).

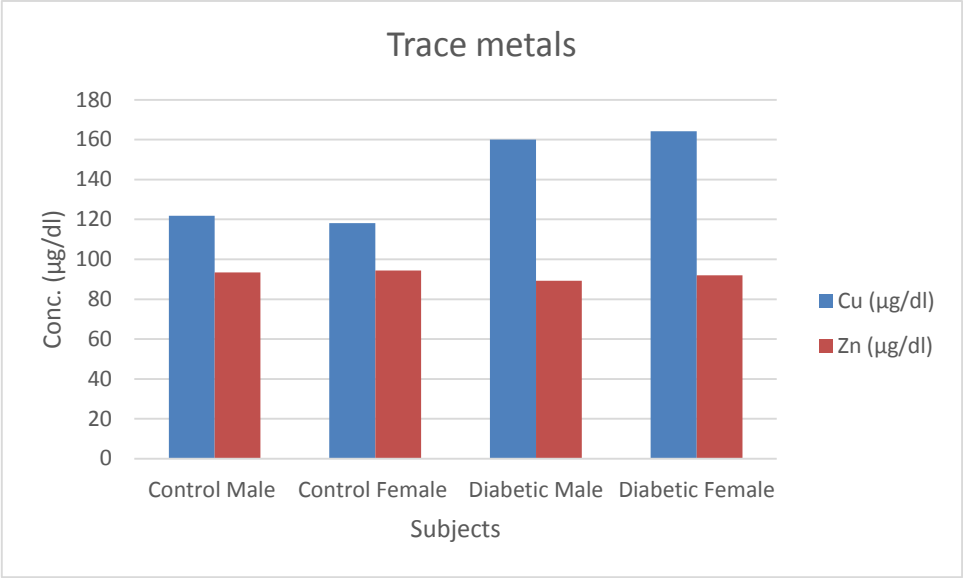


Figure 4.7: Status of trace metals according to gender

4.7 Analysis of parameters associated with iron metabolism in relation to glycated haemoglobin (HbA1c)

Table 4.11. Comparison of iron profile between Group I (healthy controls), Group II (T2DM cases with good glycaemic control), and Group III (T2DM cases with poor glycaemic control) *.

Table 4.11 Comparison of iron profile

Parameter	Group I	P value (I vs. II)	Group II	P value (II vs. III)	Group III	P value (I vs. III)
Serum Iron (µg/dl)	105.34±3.5	0.917	107.33±3.45	0.0012	125.58±3.74	0.0003
TIBC (µg/dl)	311.39±5.47	0.967	309.63±6.1	0.0015	284.2±3.18	0.0006
Tsat (%)	34.17±1.21	0.862	35.02±1.2	0.0002	44.39±1.07	0.0001

*Values expressed as mean ± SEM.TIBC: total iron binding capacity; Tsat: transferrin saturation.

As shown in Table 4.11, mean serum free iron concentration in Group I, Group II, and Group III was 105.34 ±3.5, 107.33 ± 3.45, and 125.58 ±3.74 µg/dL, respectively, while mean serum TIBC concentration in Group I, Group II, and Group III was 311.39 ± 5.47, 309.63 ± 6.1, and 284.2 ±3.18 µg/dL, respectively. Further, mean serum transferrin saturation (%) in Group I, Group II, and Group III was 34.17 ± 1.21, 35.02 ±1.2, and 44.39 ±1.07 µg/dL, respectively.

The difference between mean serum free iron concentration, TIBC, and Tsat between Group I and Group III (all three $p < 0.05$), as well as between Group II and Group III ($p = 0.0012$, 0.0015, and 0.0002, respectively) is highly statistically significant. However, the difference between these parameters in Group I and Group II is not significant ($p = 0.9178$, 0.9674, and 0.8622, respectively).

Table 4.12. Correlation of serum iron concentration with HbA1c and Tsat in Group III cases.

Parameter	Pearson coefficient (r)	P value
HbA1c	0.05	0.73
Tsat	0.496	0.732

A significant correlation was absent between serum iron and HbA1c ($r = 0.05$) and Tsat ($r = 0.0496$) in diabetic patients of Group III (Table 4.12).

4.7 Genetic polymorphism

4.7.1 MTHFR polymorphism A1298C

The A1298C (rs1801131) polymorphism has been reported to result into decreased activity of the enzyme invitro (Van der put et al., 1998, Weisberg 1998, Naomi 2001). The SNP leads to replacement of A by C at neucleotide 1298 in exon 7 resulting in a glutamate to alanine substitution (Jakubowski, 2000). While SNP C677T of MTHFR has been focus of the study in literature association of A1298C SNP is poorly understood (see section 2.10.2 for details). In order to get more insight here we studied SNP A1298C in north Indian population with respect to T2DM complications.

4.7.1.1 Genotyping study

We used allele specific PCR to detect A1298C SNP in diabetic and control DNA samples (for details of primers and PCR conditions please refer to Methods section). Based on presence or absence of 128 bp amplicon on agarose gel electrophoresis corresponding genotype was ascertained. A representative gel image was displayed in Figure4.8.

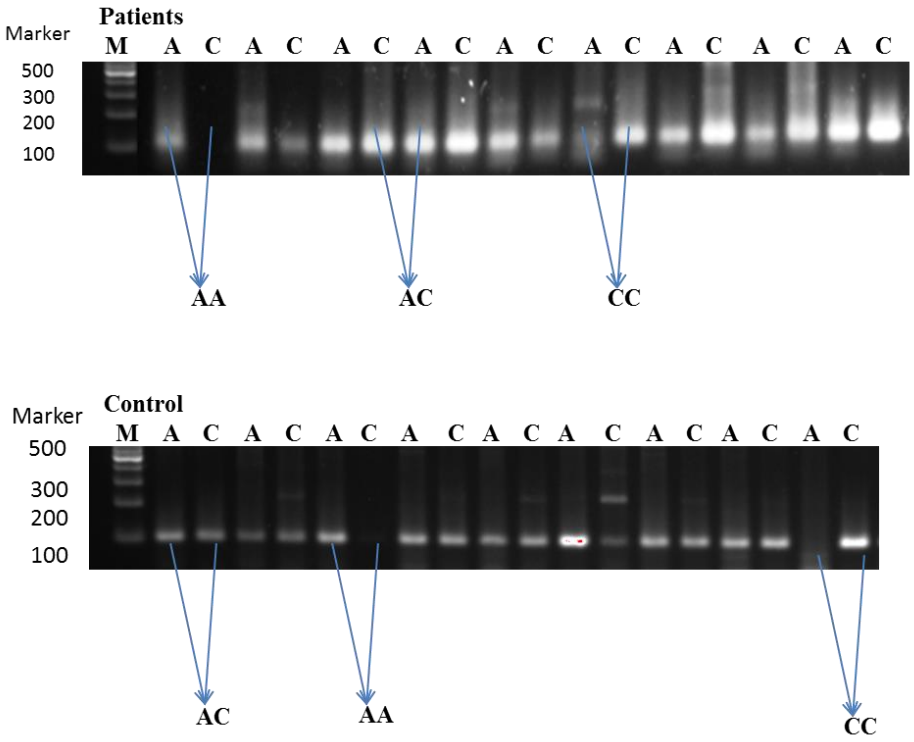


Figure 4.8: Genotyping MTHFR A1298C polymorphism-
Allele specific PCR, as labelled on top of each gel, was used to amplify 128 bp amplicon. Note that CT is prevalent genotype in patients (upper panel) while TT and CT are prevalent in controls (lower panel) see table 4. for scoring details.

4.7.1.2 Distribution of genotypic and allelic frequency and susceptibility to T2DM

Both control and diabetic samples were tested for Hardy-Weinberg Equilibrium (HWE) using χ^2 goodness of fit. No significant deviation was observed for both populations. Genotypic and allelic frequencies were presented in Figure 4.9 and Table 4.13. Genotypic frequency for AA, AC and CC was found to be 35.83%, 51.67% and 12.50% for controls while same was 30%, 55.83% and 14.7% in diabetic patients. Allelic frequencies for major and minor alleles were 61.67% and 38.33% in controls while 57.92% and 42.08% in diabetic patients. In both controls and diabetics, AC heterozygous genotype was prevalent. No significant association for risk of developing T2DM was observed for SNP allele C (OR, 1.17, 95% CI, 0.81-1.61, p = 0.403).

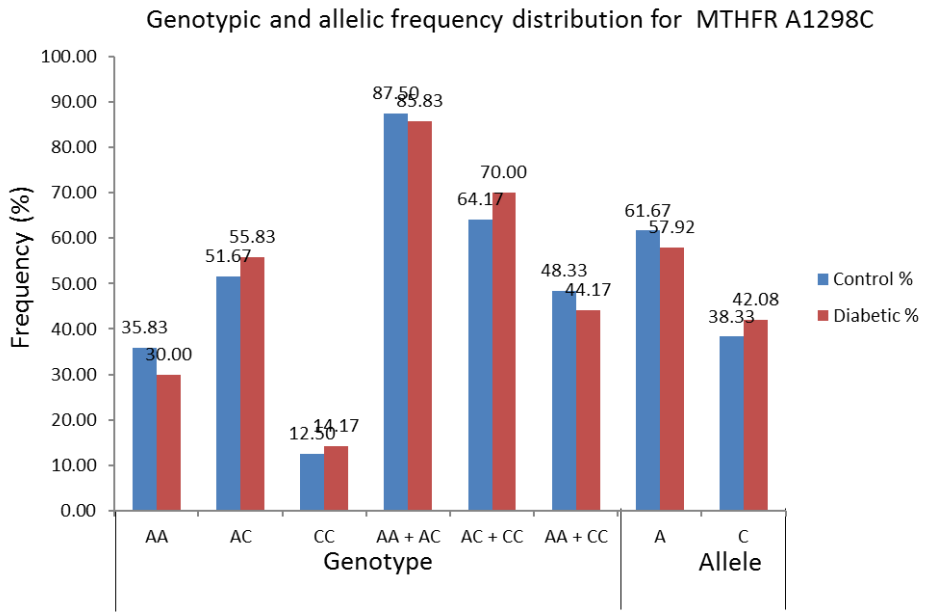


Figure 4.9: Genotypic and allelic frequency distribution of A1298C

Table 4.13: Genotypic and allelic frequency distribution

		Control (%) N=120	T2DM (%) N=120	Odd ratio at 95% CI(lower - upper)	P value	χ^2 value (Pearson)
Genotypic distribution	AA	43 (35.83)	36 (30)		0.625	
	AC	62 (51.67)	67 (55.83)			
	CC	15 (12.50)	17 (14.70)			
Allelic frequency	A	148 (61.67)	139 (57.92)	1.17 (0.81-1.61)	0.403	0.7
	C	92 (38.33)	101 (42.08)			

Further, logistic regression analysis was done using different genetic models and results were displayed in Table 4.14.

Table 4.14: Logistic regression analysis

Genetic models	Control (%) N=120	T2DM (%) N=120	Odd ratio at 95% CI(lower - upper)	P value	χ^2 value (Pearson)
AA : AC + CC (dominant model)	43 (35.83) : 77 (64.17)	36 (30) : 84 (70)	1.3 (0.76-2.24)	0.337	0.92
AA + AC : CC (recessive model)	105 (87.5) : 15 (12.50)	103 (85.83) : 17 (14.70)	1.16 (0.55-2.44)	0.708	0.14
AA : CC (co-dominant model-I)	43 (35.83) : 15 (12.50)	36 (30) : 17 (14.70)	1.35 (0.59-3.08)	0.471	0.52
AA : AC (co-dominant model-II)	43 (35.83) : 62 (51.67)	36 (30) : 67 (55.83)	1.29 (0.74-2.26	0.374	0.79

As evident from Table 4.14, SNP A1298C was not significantly associated with risk of developing T2DM in dominant model (OR, 1.3, 95% CI, 0.76-2.24, p = 0.337), recessive model (OR, 1.16, 95% CI, 0.55-2.44, p = 0.708), co-dominant model-I (OR, 1.35, 95% CI, 0.59-3.08, p = 0.471) and co-dominant model-II (OR, 1.29, 95% CI, 0.74-2.26, p = 0.374).

4.7.2 TGFβ1 polymorphism T869C

Recently, association between T869C polymorphism and progression of T2DM have been reported in different populations (Sherif et al., 2013; Teresa et al., 2003). This SNP includes replacement of proline for leucine at 10^{th} amino acid. The secretory function of former is almost twice that of later allele (Dunning et al., 2003). It has been suggested that T allele display some sort of protective nature while C allele is the susceptible culprit behind T2DM occurrence (Sherif et al., 2012). Inerestingly, a recent study reported that the CC genotype of this SNP elevates the risk of nephropathy 3.1 to 4.5 times in Indian population (Raina et al., 2015). Thus, it was quite reasonable to test

whether SNP T869C is associated with risk of T2DM development in north Indian population.

4.7.2.1 Genotyping study

We used allele specific PCR to detect T869C SNP in diabetic and control DNA samples (for details of primers and PCR conditions please refer to Methods section). Based on presence or absence of 241 bp amplicon on agarose gel electrophoresis corresponding genotype was ascertained. A representative gel image was displayed in Figure4.10.

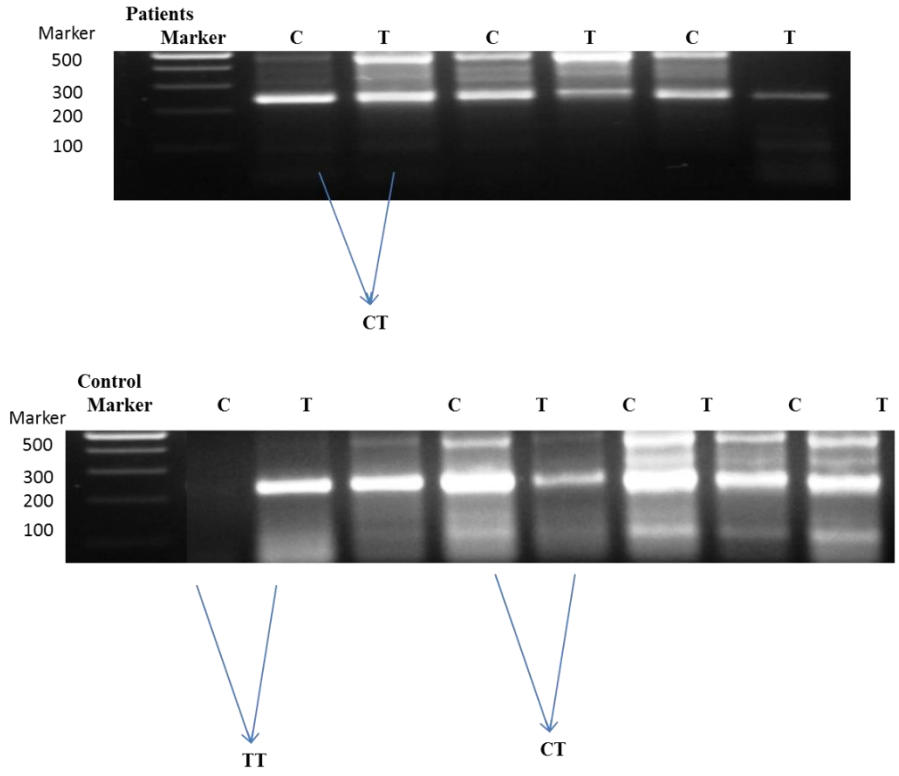


Figure 4.10: Genotyping TGF β1 T869C polymorphism-
Allele specific PCR, as labelled on top of each gel, was used to amplify 241 bp amplicon. Note that CT is prevalent genotype in patients (upper panel) while TT and CT are prevalent in controls (lower panel) see table 4. for scoring details.

4.7.2.2 Distribution of genotypic and allelic frequency and susceptibility to T2DM

Both control and diabetic samples were tested for Hardy-Weinberg Equilibrium (HWE) using χ^2 goodness of fit. No significant deviation was observed for both populations.

Genotypic and allelic frequencies were presented in Figure 4.11 and Table 4.15. Genotypic frequency for AA, AC and CC was found to be 70%, 26.67% and 3.33% for controls while same was 39.17%, 51.67% and 9.17% in diabetic patients. Allelic frequencies for major and minor alleles were 83.33% and 16.67% in controls while 65% and 35% in diabetic patients. TT homozygous genotype was prevalent in controls while in diabetics TC heterozygous genotype was prevalent. Significant association for risk of developing T2DM was observed for SNP allele C (OR, 2.69, 95% CI, 1.75-4.14, $p < 0.05$).

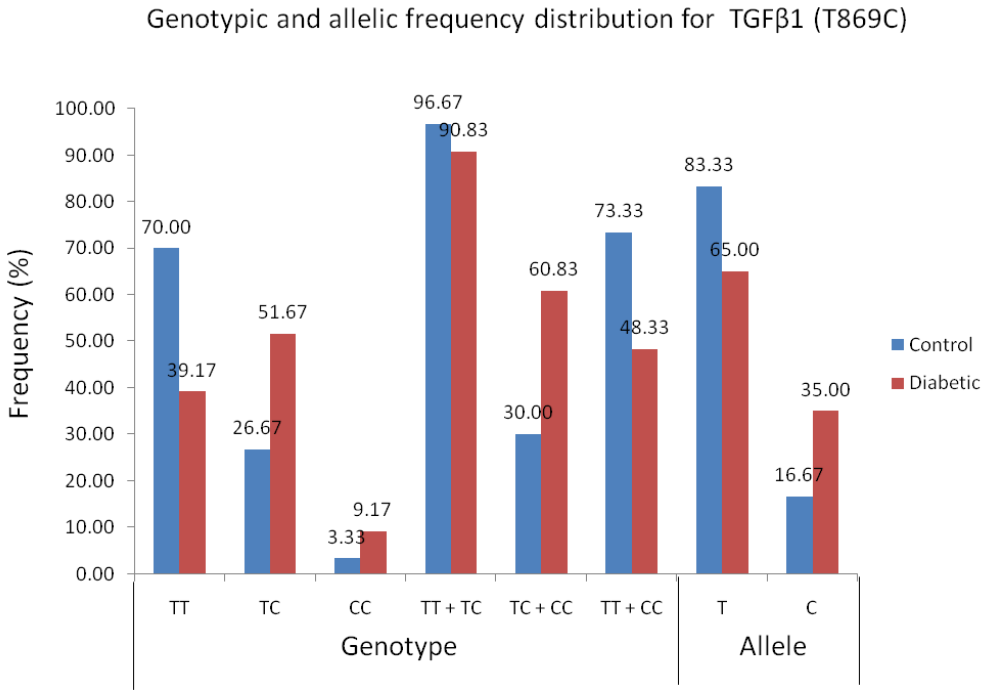


Figure 4.11: Genotypic and allelic frequency distribution of T869C

Table 4.15: Genotypic and allelic frequency distribution

		Control (%) N=120	T2DM (%) N=120	Odd ratio at 95% CI(lower - upper)	P value	χ^2 value (Pearson)
Genotypic distribution	TT	84 (70)	47 (39.17)		<0.05	
	TC	32 (26.67)	62 (51.67)			
	CC	4 (3.33)	11 (9.17)			
Allelic frequency	T	200 (83.33)	156 (65)	2.69 (1.75-4.14)	<0.05	21.05
	C	40 (16.67)	84 (35)			

Further, logistic regression analysis was done using different genetic models and results were displayed in Table 4.13.

Table 4.16 Logistic regression analysis

Genetic models	Control (%) N=120	T2DM (%) N=120	Odd ratio at 95% CI(lower - upper)	P value	χ^2 value (Pearson)
TT : TC + CC (dominant model)	84 (70) : 36 (30)	47 (39.17) : 73 (60.83)	3.62 (2.12-6.19)	<0.001	23.01
TT + TC : CC (recessive model)	116 (96.67) : 4 (3.33)	109 (90.83) : 11 (9.17)	2.93 (0.9-9.47)	0.062	3.48
TT : CC (co-dominant model-I)	84 (70) : 4 (3.33)	47 (39.17) : 11 (9.17)	4.91 (1.48-16.3)	0.005	7.89
TT : TC (co-dominant model-II)	84 (70) : 32 (26.67)	47 (39.17) : 62 (51.67)	3.46 (1.98-6.04)	<0.001	19.83

As evident from Table 4.16, SNP T869C was not significantly associated with risk of developing T2DM in recessive model (OR, 2.93, 95% CI, 0.9-9.47, p = 0.062) while it displayed significant association in dominant model (OR, 3.62 95% CI, 2.12-6.19, p

<0.05), co-dominant model-I (OR, 4.91, 95% CI, 1.48-16.3, p = 0.005) and co-dominant model-II (OR, 3.46, 95% CI, 1.98-6.04, p <0.001).

PTPN22 1858 C/T SNP

No polymorphic bands were observed after digestion of PCR product with XcmI and RsaI restriction enzymes in controls as well as patients therefore the loci PTPN C/T was found to be non-polymorphic and is not associated with type 2 diabetes in our population.

4.8 ^{1}H-NMR based serum metabolic profiling

4.8.1 Metabolite Assignment

A typical 1D ^{1}H CPMG NMR spectra of serum samples obtained from different groups are shown in Figure 12. The NMR spectra showed signals mainly from lipids/lipoproteins (e.g. low density lipoprotein (LDL), very low density lipoprotein (VLDL), polyunsaturated fatty acids (PUFAs) etc.), membrane metabolites [e.g. choline, phosphocholine (PC), and Glycerophosphocholine (GPC)], N-acetyl glycoproteins (NAG), and amino acids (e.g. leucine, isoleucine, valine, alanine, lysine, proline, glutamine, glutamate, histidine, tyrosine, and phenylalanine etc.). Other identified metabolites were, glucose, lactate, acetate, citrate, creatine/creatinine.

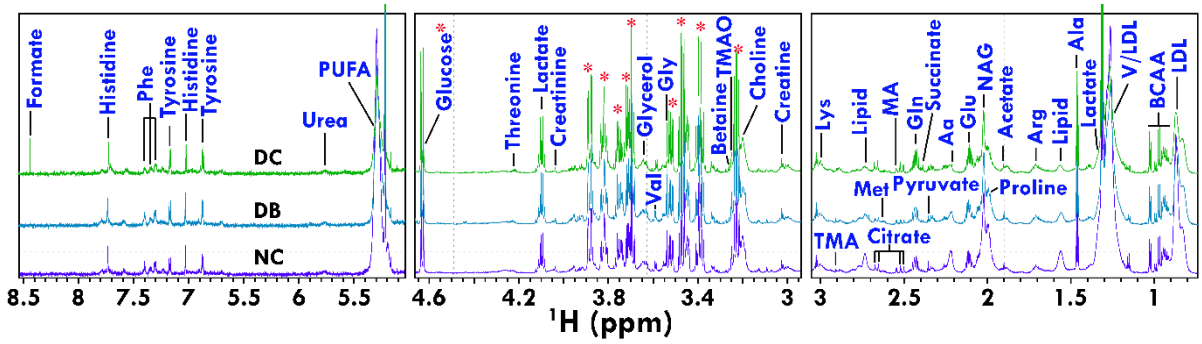


Figure 12: Stack plot of representative 1D ^{1}H NMR spectra of serum obtained from different groups

The peaks annotated in the figure show the assignments of serum metabolites. The abbreviations used are LDL/VLDL: Low/very-low-density lipoproteins; PUFA: polyunsaturated fatty acids; BCAA (Branched chain amino acids): Isoleucine; Leucine; Valine; Aa: Acetoacetate; Lys: Lysine; Phe: Phenylalanine; Gly: Glycine; Val: Valine; Gln: Glutamine; Glu: Glutamate; Arg: Arginine; Ala: Alanine; Met: Methionine; MA: Methylamine; TMA: Trimethylamine.

4.8.2 Supervised classification

Random Forest was used for multivariate analysis on the 1D ^{1}H NMR datasets. RF is a powerful supervised classification method available in statistical analysis module of MetaboAnalyst. It uses an ensemble of classification trees, each of which is grown by random feature selection from a bootstrap sample at each branch. Class prediction is based on the majority vote of the ensemble. During tree construction, about one-third of the instances are left out of the bootstrap sample. This data is then used as test sample to obtain an unbiased estimate of the classification (OOB = out of the bag) error. RF analysis was performed to get a discriminatory overview of the three cohorts and further pair wise analysis was also performed between the groups to identify the differentiating metabolites i.e DB vs NC, DC vs NC and DCvs DB as shown in Figure 13.In RF analysis500 trees were grown and 7 features wererandomly selected at each node. The generalization error wasestimated on the OOB samples.

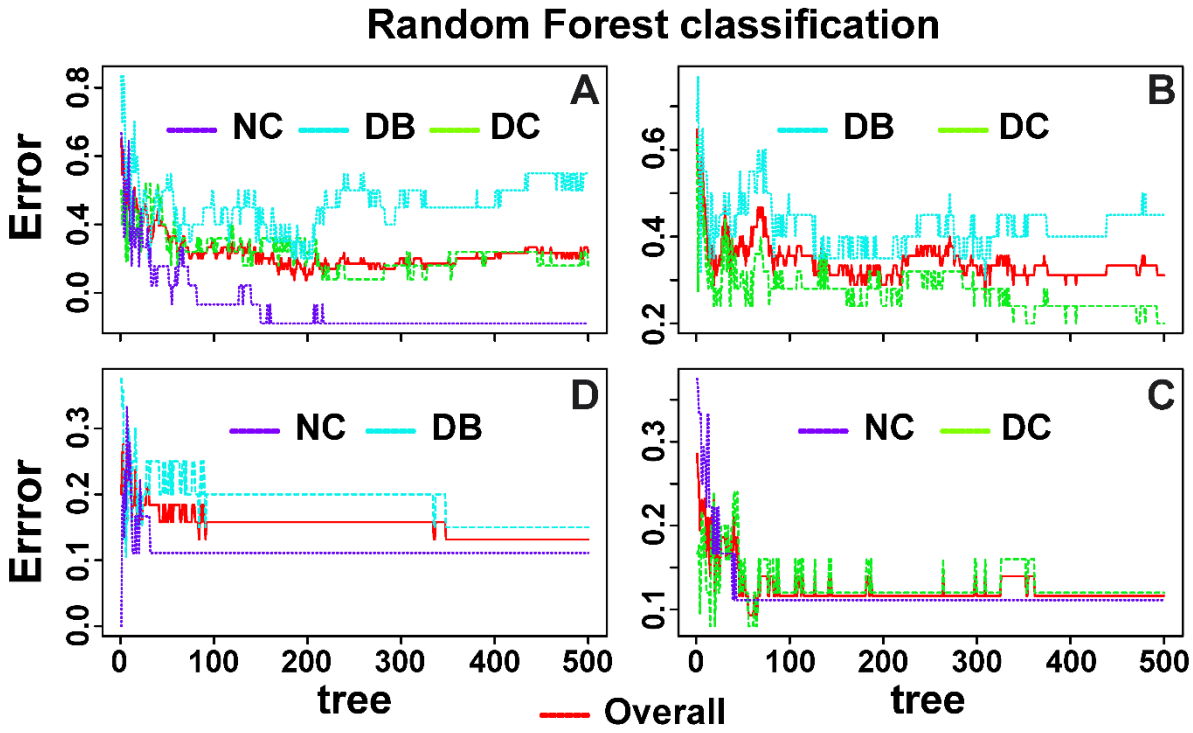


Figure 13:Random Forest classification

RF analysis of 1D ^{1}H CPMG NMR spectra comprising of all the groups(**A**) and pair wise analysis (**B, C, D**).

The overall error rate is shown by the red line,the respective colours show the error rates for each class. Next we determined the important biomarkers in metabolomics dataset of pair wise analysis. Variable importance (VI) is based on MDA, which is the result of the permutation of the average over all trees and is used to measure the importance of the variables in RF. After manual data mining and processing, we identified metabolites significantly perturbed in sera of different cohorts (MDA ≥0.01) as enlisted in Table 4.14. The quantitative difference in the metabolite concentration was assessed using t- test, (p-Value< 0.05). The discriminatory metabolites were mainly related to lipid, amino acid, glucose, and energy metabolism. Further receiver's operating characteristic (ROC) curves analysis was performed for the significant metabolite markers to evaluate their predictive power or diagnostic accuracy. The area under the ROC curve gives of discriminatory ability (0.5=no discrimination; 1=perfect discrimination). The largest and smallest resulting AUC values range from 0.98 to 0.52 (Table 4.15) which indicated that these metabolites could be potential biomarkers for

diagnosis, surveillance (through parallel assessment of a wide range of metabolites), and early detection of metabolic perturbations insuch patients.

The rise and fall of the metabolites in body fluids can distinguish between healthy and diseased states. Compared with those from normal controls, serum samples of patients with DB and DC showed (a) increased levels of glucose, methionine, histidine (b) by the decreased levels of LDL/VLDL, lipids, polyunsaturated lipids, N-acetyl glycoproteins(NAG), lipids, lactate, pyruvate, citrate, succinate, acetoacetate,creatinine/creatine,and most of the amino acids (like alanine, valine, isoleucine, proline, glutamate, glutamine, arginine, histidine, and phenylalanine, etc). Most of the metabolites in DB and DC followed a similar pattern in matter of their increased or decreased levels in serum with respect to the control group. However, when compared to the DB, the DC patients have an increased levels of succinate, citrate, glucose, threonine, PUFA and urea by the decreased levels of LDL/VLDL, lipids, N-acetyl glycoproteins(NAG), pyruvate, creatine, and amino acids (like valine, arginine, glutamate, methionine, and proline. The results clearly shows that protein biosynthesis, amino-acid metabolism, glucose-energy metabolism and glycerolipid metabolism are disturbed in patients with DC. Hence metabolic perturbation in patients with DC has been discussed in detail in discussion part.

4.9 **General Knowledge and awareness about diabetes**

Table 4.17 and 4.18 shows the results on general awareness, knowledge about diabetes, its symptoms and complications. Only 63 patients (52.5%) knew that they suffered from type 2 diabetes, the characteristic feature of which is elevated blood sugar level. 26 patients (21.66%) were aware that this was a consequence of defective insulin mechanism. 65 patients (54.2%) were mindful of the fact that type 2 diabetes can be prevented and controlled. 61 patients (50.8%) were acquainted with the connection of

T2DM with family history. 82 patients (68.33%) recognized obesity as a risk factor for T2DM while 74 (61.66%) of them were familiar with sedentary behaviour being a culprit in causing diabetes.

Regarding source of information about diabetes, 81 patients (67.5%) confirmed acquiring it from their attending physician. 54 patients (45%) acknowledged their family and relatives and 28 (23.33%) admitted of getting the information from their friends and neighbours. 21.66% patients owned about getting information from print media while 10.83% did so with the help of electronic media.

Frequent urination was found to be the most commonly recognized symptom among the patients (n=70, 59.4%). It was followed by frequent thirst reported by 53 of them (43.03%). 45 patients (37.5%) were conversant with frequent hunger as a symptom and abnormal weight gain/loss was recognized as a symptom of diabetes by 31 (25.83%) patients. Only 15 patients (12.5%) were well versed with the fact that diabetes can be asymptomatic.

The most commonly recognized diabetic co-morbidity was heart problem as reported by 71 patients (59.2%), followed by eye problems recognized by 53 patients (44.24%) and kidney complications by 50 patients (41.66%). 40 patients (33.33%) were alert to foot problems while 21 of them (17.5%) recognized neurological problems as a diabetic complication. 14.16% patients reported other problems related to diabetes.

Factors related to non-adherence to diabetes treatment, affecting the ideal patient outcome are listed in table 3. 34 patients (28.33%) confirmed that they sometimes forgot to take their medicines. 37 patients (30.8%) complained of side effects with their prescribed medicines. 17 patients (14.16%) confessed of skipping their medicines when they felt their symptoms to be under control. 44 of them (36.66%) expressed concern over their disease status and were stressed by it. 45 (37.5%) of them were dissatisfied

with the interaction with their physicians. A majority of patients 88 (73.33%) confided that the treatment was a burden on their economic resources. 36 patients (30%) admitted to following alternative forms of therapy ex, homeopathy, ayurved etc.

Table 4.17 -General Knowledge and awareness about diabetes, its symptoms and complications

General awareness about diabetes and its risk factors	**No. Of correct responses (%)***
Knowledge about type of diabetes	63 (52.5)
Abnormal insulin levels cause diabetes	26 (21.66)
Diabetes can be prevented and controlled	65 (54.2)
family history is a risk factor for diabetes	61 (50.80)
Obesity is a risk factor for diabetes	82 (68.33)
Sedentriness is a risk factor	74 (61.66)
Source of information about diabetes	
Attending physician	81 (67.5)
Family and relatives	54 (45)
Friends and neighbours	28 (23.33)
Electronic media	13 (10.83)
Print media	26 (21.66)
Knowledge about diabetes symptoms	
Abnormal weight gain/loss	31 (25.83)
Frequent urination	70 (58.33)
Frequent thirst	52 (43.33)
Frequent hunger	45 (37.5)
Asymptomatic	15 (12.5)
Knowledge about diabetic complications	
Heart problems	71 (59.2)
Kidney problems	50 (41.66)
Eye diseases	53 (44.16)
Foot infections	40 (33.33)
Neurological disorders	21 (17.5)
Others	17 (14.16)

*multiple answers by a participant regarding knowledge source, symptoms and complications were acceptable.

Table 4.18- factors related to non-adherence affecting ideal patient outcome

Questions	**No. Of positive responses (%)**
Are you forgetful about your medicines sometimes?	34 (28.33)
Do you experience any side effects with the prescribed medicines?	37 (30.8)
Do you sometimes skip medicines when you feel your symptoms are under control?	17 (14.16)
Do you feel stressed out at your disease status?	44 (36.66)
Are you dissatisfied with your interaction with the physician?	45 (37.5)
Do you face economic crunch in continuation of your treatment?	88 (73.33)
Do you follow any alternative form of treatment (homeopathy, ayurvedic etc.)?	36 (30)

Chapter 5:

DISCUSSION

DISCUSSION

Diabetes mellitus is a persistent metabolic disorder that is plaguing populations worldwide. Type 2 diabetes mellitus (T2DM) constitutes more than 95% of all the cases. India is one of the hubs of the universal diabetes mellitus pandemic and ranks second in the world in terms of sheer number of people affected by its (about 69 million people as of 2015) (IDF Diabetes Atlas, 2015). Asian Indian community which broadly encompasses the individuals from the Indian subcontinent (natives of India, Sri Lanka, Bhutan, Pakistan, Nepal, Bangladesh, Afghanistan and the Maldives) (United Nations Geographic Region Classification, 2016) represent more than 17% of the total population of world and possess a particular phenotype, distinguished by elevated measures of fat in intra-abdominal section plus insulin resistance despite having a low BMI and makes them vulnerable towards developing T2DM and other vascular diseases (Unnikrishnan et al., 2014). To gain insights into the frame work and action of T2DM in this ethnic group is the utmost priority of today's times. Luckily, with the rising prevalence of diabetes in India, research work on the disease and its complications has also increased. Although there is now superior understanding about the various facets of T2DM, its multi-factorial pathogenesis still leaves us with more questions than answers. Various publications have suggested that oxidative stress which is the progenitor of free radicals; together with the collapse of the inherent antioxidants are the main offenders which pave the way for the evolution and advancement of critical diabetic complications (Maritim et al., 2003; Ceriello, 2000). T2DM is one of those complex diseases the genetic contribution of which is well acknowledged. Recognising genetic elements of T2DM is one of the foremost areas of diabetes research because clarification of genes and alleles involved in diabetes and its complications will go a long way in understanding the mechanism behind its aetiology, complications, possible

treatment and cure and most importantly its prevention. Furthermore, last decade has witnessed the rapid evolution of metabolomics and its application in studies related to diabetes. This new technique enables the researchers to gain insights into the metabolic profiling of the diseased individuals and to identify novel biomarkers which would smooth the way for further understanding of the pathophysiological mysteries of T2DM. Thus, a combined approach involving biochemical, genetic and metabolomic aspects towards understanding the pathos behind T2DM along with its societal implications is the need of the hour.

5.1 Anthropometric features of the subjects

BMI

Obesity is one of the major risk factors for diabetes, yet there has been little research focusing on this risk factor across India (Rao et al., 2011). Despite having lower overweight and obesity rates, India has a higher prevalence of diabetes compared to western countries suggesting that diabetes may occur at a much lower body mass index (BMI) in Indians compared with Europeans (Mohan and Deepa, 2006; Rao et al., 2011). Significantly higher values ($p<0.001$) were observed in our study in diabetics as compared to controls. The increasing incidences of obesity may result in more than a million extra cases of type 2 DM, cardiovascular disease and cancer.

Socio-economic and demographic feature of the study subjects.

Although the causal routes between socio-economic position and type 2 diabetes are yet not well defined, it may contribute towards T2DM development via complex pathways involving availability of healthy foods, places for physical activity, economic and occupational opportunities as well as life style choices on an individual basis (Brown et al, 2004).

Age

Age is a crucial risk factor for T2DM (Anjana et al, 2011) and Asian Indians are reported to have a tendency of developing it at a comparatively young age than Caucasians (Ramachandran et al., 2010). It has been postulated that the reason behind this could be the earlier onset age of significant weight gain seen in young adults plus genetic factors (Boffetta et al., 2011).

This study has shown that the population most affected by diabetes is of the age group 46-60. This is corroborated by another study in district Sonepat of Haryana state (Madaan et al., 2014). This is a disturbing finding as pervasion and persistence of diabetes in the most economically productive age group means staggering economic growth of society.

Marital status

A study has shown that being married may confer health advantage against type 2 DM as against bachelors, divorcees and widowers (Cornelis et al, 2014). Similar significant trend ($p<0.0015$) is seen in our study proving that it is a key support mechanism for the subjects.

Occupation

According to census of 2001, work force in India consisted of >400 million individuals, comprising of about 40% of the total population of the country. This economically productive age group spends majority of their time at their work place with an average of about 48 hours per week (Brownson et al., 2005). The connection of duration of sitting for >3 hours at workplace enhances the risk of T2DM. Our results follow similar trend as about 50.83% of our diabetic subjects were service men who were in habit of working long hours at their desks in their work place.

Smoking

Smoking constitutes one of the reversible or modifiable risk factor for various chronic diseases, such as diabetes, Cancer, chronic obstructive lung disease, Cardio vascular disease and asthama. But the detrimental effects of smoking on T2DM are generally side-lined. It is associated with central obesity (Canoy et al., 2005), inflammation and oxidative stress (Morrow et al., 1995), damage of ß cell function (Spector and Blake, 1988) and impairment of endothelial function (Noma et al., 2005).

Majority of the diabetic patients in the study being non-smokers points towards adoption of a healthier lifestyle in view of their diseased state.

Drinking

A study has reported that the relationship between alcohol intake and glycaemic control is inversely proportional thus concluding that diabetic co-morbidities can be curtailed by reducing alcohol consumption (Ahmed et al., 2008). Most of the diabetic patients claimed to be non-drinkers and this again points towards a positive lifestyle intervention.

Physical Activity

Physical activity or exercise has impact on various components of diabetes. Ever increasing urbanization and socio-economic prosperity has led to decline in the physical activity levels of people. Statistically significant values (p<0.0089) from our study indicates the same and is corroborated by many other findings (Bhatti et al., 2007; Hughes et al., 1990; Lip et al., 1996; Williams et al., 1994) which have shown that South Asians and Asian Indians are lesser physically active than other ethnic groups. It is interesting to note that in our study most of the controls as well as patients seem to follow a mild exercise routine. This resistance towards strenuous physical activity tips the balance in favor of strong insulin resistance in diabetics and may pave the way

towards impaired glucose tolerance in controls later on. This agrees with a south Indian study which has shown that diabetes prevalence is almost three times higher in individuals with light physical activity compared to those having heavy physical activity (Mohan et al, 2003). Environmental barriers responsible for limited physical activity include unsafe walking areas, transportation problems, medical conditions and also the attitudes and knowledge of subjects (Dutton et al., 2005).

Cooking medium

Edible oils constitute an important part of diet of a person. A study on the effect of edible oils on biochemical parameters of subjects in Kharagpur, west Bengal shows that sesame oil followed by mustard oil proved to be most beneficial against diabetes (Kumar et al, 2009). Our findings show that use of refined oil is more prevalent than mustard oil and the difference in consumption pattern is significant ($p<0.0079$). It is perhaps advisable to revert to our traditional dietary ways to reduce the incidence of diabetes.

Consumption of junk food

The role of junk food in diabetes is highlighted by a study on Musmusculus albinus mice, which were exclusively fed junk food for thirty days. Their body weight and blood sugar levels clearly indicated that fast food enhanced the risk of obesity and diabetes (Wast et al., 2012). Our study points out that most of the diabetics (about 60%) indulge in fast food sometimes while 47.5% of healthy controls do so and the difference was significant ($p<0.0232$). An interesting contrast is seen when controls are shown to be frequent consumers (48.3%) as against diabetics (36.6%). This can be attributed to the fact that once the disease is diagnosed, patients tend to make healthier food choices.

Family history of type 2 diabetes mellitus in the study subjects

About 67% of diabetic subjects had either one or both parents affected by diabetes. Upon analysis, a highly significant association ($p<0.0001$) between family history and T2DM was observed. Similar results were reported by other studies (Patil and Gothankar, 2013; Ramchandran et al., 2008; Rao et al., 2010; Ravikumar et al., 2011; Shah et al., 1999). Family history in T2DM is thus a major risk factor in transferring the disease to next generation. It can however be exploited as a preventable tool to avoid diabetes development in early age.

5.2 Biochemical Studies

Different biochemical tests were performed and the results of these tests aimed at detecting the presence and extent of any type of co-morbidity.

Glycated Haemoglobin

Glycated hemoglobin (HbA1C) is a routinely used marker for long term glycemic control. In accordance with its function as an indicator for the mean blood glucose level, HbA1C predicts the risk for the development of diabetic complications in diabetic patients.

MDA

The study revealed elevation in serum MDA concentrations in diabetic patients as compared to controls. This is in line with various previous studies. Enhanced non-enzymatic glycosylation and auto oxidation of glucose may be the reason behind exhaustive generation of free radicals which incite lipid peroxidation and as a result enhanced MDA concentration. There was significant positive correlation observed in the study observed between MDA levels and indices of glycaemic control i.e., fasting plasma glucose and HbA1c values. Similar findings have been reported by other studies (Noberasscoet al., 1991).

Lipid Profile

Nowadays, the term "hyperlidaemia" is increasingly being replaced by "Dyslipidaemia" to explain alterations in blood lipid levels. It involves changes in HDL, TC/HDL ratio and size and density of LDL (Goldberg, 2001). Diabetic dyslipidaemia includes the triad of elevated triglycerides, an excess LDL and diminished HDL levels (O'Brien et al., 1998; Colhoun et al., 2004). Lipid profile in this study shows the same trend. It is suggested that lipid composition in diabetic dyslipidaemia is atherogenic to a greater degree than dyslipidaemia of other types. This indicates that normal lipid levels may also tend to more atherogenic in diabetic patients as compared to non-diabetic individuals (Taskinen, 2002). Atherosclerosis is responsible about 80% mortality in diabetic patients due to coronary heart disease and peripheral or cerebrovascular disease.

The significantly high levels of TG, TC and low levels of HDL-c observed in the study subjects probably contribute to insulin resistance (Mooradian, 2009). However, lower HDL values observed in diabetic patients attending primary care is now well known (Grant and Meigs, 2007). This high prevalence of hypertriglyceridemia, high LDL, low HDL and hypercholesterolemia in the diabetic patients are known risk factors for cardiovascular disease. It is in line with another study done at Naini region of Allahabad which concluded that Hypercholesterolemia, Hypertriglyceridaemia and lipoprotein are the main lipid abnormalities found in diabetes which are risk factors for coronary artery disease (Smith and Lall, 2008). This is validated by our study as about 24% of our patients were found to be hypertensive (refer to table), and were thus at risk of developing Coronary Heart disease which is a major co-morbidity attached with diabetes. American Diabetes Association (ADA) has also discussed about the rationale for management of dyslipidaemia in Adults with diabetes (Haffner, 1998).

The significant correlation observed between lipid parameters and fasting plasma glucose levels are in accordance with previous studies (Ito et al., 2000; Rosediani et al., 2006).

The lipid profile of the diabetic patients in the study did not show significant correlation with either age or diabetes duration. These results are corroborated by other studies which have reported that neither age group of subject nor the duration of diabetes had any significant effect on the lipid profile (Ochei and Kolhatkar, 2000; Otamereet al., 2011). Studies showing contrasting results have also been reported (Bucala et al; 1993; Lyons, 1993;Ramirez et al., 1992;Schwart et al., 1992). These studies have hinted at the aberration in vascular functional integrity in diabetes mellitus and indicated that the aberration is dependent on diabetes duration. Age has also shown to be affecting the lipid levels (Estari et al 2000). But, it should be considered that the diabetic subjects in this study were on oral hypoglycemics. Such treatment not only reduces the glucose levels in blood, but also affects the entire pathophysiology including the lipid profile. Thus age and diabetes duration may not be powerful indices for prediction of lipid profile status in diabetic subjects under medication/management (Bucala et al; 1993; Estari et al., 2000; Lyons, 1993;Ramirez et al., 1992;Schwart et al., 1992).

Uric Acid

In the present study, it was observed that serum UA level was significantly increasing (p<0.001) in T2DM patients as compared to controls. This finding is concurrent with other studies in which hyperuricemia is linked to development of T2DM and its complications especially cardiovascular (Zoppini et al., 2009) and renal complications (Bo et al., 2001; Rosolowsky et al., 2008). Uric acid is a strong endogenous antioxidant that scavenges nitric oxide directly thus decreasing the bioavailability of nitric oxide in vascular smooth muscles and endothelial cells. This promotes endothelial dysfunction

enhancing the risk of progression of Coronary vascular disease (Conen et al, 2004; Feig et al, 2008).

Creatinine

Serum creatinine levels in T2DM patients were also significantly higher when compared statistically with controls ($P < 0.05$) indicating the derangement of kidney function. It is believed that one can plot the inverse of creatinine (I/ Cr) over time and get a straight line which can thus be used for "monitoring disease progression"(Mitch and Walser, 1986). A study on progression of nephropathy in T2DM pointed out that T2DM is single most common cause of end stage renal disease (ESRD), but decline in kidney function varies among individuals (Kasper et al., 2004). These findings are further corroborated by a retrospective analytic study, conducted by reviewing the clinical records of the patients with type 2 diabetes who attended the National Diabetes Centre of Sri Lanka from January 2005 to December 2010 it was observed that nephropathy was significantly associated with poor glycemic control, high HbA1c, high fasting blood glucose, high systolic blood pressure (Wijesuriya et al., 2012). Hence the pathogenesis of diabetic nephropathy is multi-factorial with contribution from various metabolic abnormalities, hemodynamic alterations, and marked heterogeneity in clinical picture is seen in long-term diabetics.

Prevalence of Metabolic Syndrome among the diabetic patients

Upon observing, the overall prevalence of MS was found to be 80% among the diabetic patients. This can be accredited to the fact that type 2 diabetics already are more prone to developing MS since T2DM in it-self is a diagnostic component of the syndrome. This is in accordance with another study that showed prevalence of 70-80% among Caucasian T2DM patients (Abdul-Rahim et al., 2001). Moreover, studies that have used the harmonized definition have reported high prevalence of MS affirming its role in

improved diagnosis of the syndrome in other diabetic populations also (Ogbera, 2010; Tan et al., 2013).

Moreover, majority of our MS affected diabetic subjects showed a congregation of three and/or four components. This type of assemblage is a frequently occurring event among T2DM patients, which appreciably enhances the risk of CVDs (Tong et al, 20007). These patients were thus in high CVD risk zone surpassing their fellow diabetic patients without MS. They all need special attention in terms of rigorous risk factor management and life style modifications to reduce the risk of cardiovascular co morbidities later in life. Thus, these observations helped in charting out the scenario of MS among the diabetic patients.

Status of antioxidant enzymes in the study subjects

The overall effect of T2DM on the antioxidant levels is a complicated affair as diabetes attacks individual systems of the human body at the same time. Thus studying the status of various enzymes participating in different yet interconnected pathways was necessary.

Superoxide Dismutase (SOD)

In the present study, SOD activity was observed to be increased in diabetic patients as compared to controls. Similar results have been reported by previous study (Moussa, 2008) who observed that activity of SOD in erythrocytes showed elevated activity in diabetics and enhanced levels of MDA suggesting an increased production of free radical species in such patients. High antioxidant capacity has also been reported by other studies (Savu et al., 2012). Despite the elevation in SOD activity, the existence of oxidative damage could be described by differential cellular responses to oxidative stress (Halliwell and Gutteridge, 2007). These may be associated with exhaustion of antioxidant defences as well as with increased generation of free radicals such as

superoxide radical, existence of toxins, uncurbed activation of reactive species like phagocyte activation in long standing inflammatory diseases as seen in diabetes mellitus (Halliwell and Gutteridge, 2007). Thus the enhanced activity levels observed in the present study indicate a feasible adaptive retort, probably due to increased generation of superoxide ion which would in turn accelerate the production of H_2O_2. Such condition would probably command a higher Catalase or GPx activity. But, in the present study the activities of both of these enzymes were significantly decreased in patients as compared to controls. Hence, the elevated SOD/CAT or SOD/GPx ratio in diabetic patients versus controls hinted at imbalance between SOD and GPx as well as between SOD and catalase activities which in turn would indicate the increased H_2O_2 production. $H_2O_2$when present in excess concentrations had been connected with pancreatic beta cell lesions causing alterations in cell signalling as well as gene expression (Maechler et al., 1999). Simultaneous decrease in plasma GPx levels and increase in plasma SOD levels has been reported earlier (Palanduz et al., 2001).

This SOD activity when seen in light of altered lipid per-oxidation suggests that the lack of glycaemic control even with the help of medication may alleviate oxidative stress via activation of NADPH oxidase subsequently enhancing superoxide ion concentrations. It is worth noting that the study patients were using oral anti-diabetic agents, but oxidative damage was still evident, hence the medication was not adequate to prevent oxidative stress from occurring. Moreover, according to a previous study (Wittmann and Nagy, 1996), excess generation of superoxide ions can diminish the role of nitric oxide in insulin signalling system and thus encourages the expression of insulin resistance in diabetic subjects. The consequences of insulin on SOD activity have been explored and it has been proposed that enhanced sensitivity and secretion towards insulin averts non-enzymation glycation of SOD and boosts the elevation in its activity which in turn

neutralizes superoxide ions, thus curtailing the results of insulin resistance caused by oxidative stress. In the present study SOD was positively correlated with the levels of FPG and HbA1c but these correlations were not significant.

Glutathione peroxidise (GPx)

The study shows decreased activity levels of enzyme GPx in diabetic patients as compared to controls which may be attributed to the low glutathione content responsible for low activity of GPx as reported by a previous study (Nagasaka et al., 1989). However, glutathione estimation was not carried out in this study. GPx is a relatively stable enzyme in normal conditions but under persistent oxidative stress, it may be inactivated (Condell and Tappel, 1983). This inactivation of GPx would cause accumulation of H_2O_2 in diabetic patients and would eventually decrease the activity of SOD in later stages of the disease. Results of previous studies have shown that excessive generation of peroxides with dwindling antioxidant defense leads to oxidative damage and such events are observed in T2DM patients before the development of full blown complications (Kumawat et al., 2009; Mahreen et al., 2010; Ohtsuki et al., 1995; Salem et al., 2011). Decreased GPx activity is associated with increased correlation between the enzyme and BMI. However, the correlation analysis between GPx activity and BMI of diabetic subjects of the present study showed weak negative correlation which was not significant.

Glutathione Reductase (GR)

Glutathione reductase (GR) activity levels were found to be increased in the study patients as compared to controls. The levels of GR and GPx are crucial in maintaining glutathione homeostasis in the body. Inverse relation was seen between the activity levels of these two enzymes in this study. Similar results between GPx and GR in patients of T2DM was reported by a previous study (Kumawat et al., 2005). Another

study reported simultaneous decrease in the activity levels of GPx and GR (Kornhauser et al., 2008). Enhanced activity of GR may be a compensatory response towards oxidative stress. Alterations in the activity of these two primary enzymes responsible for maintaining glutathione levels in the body can be considered an adaptation of antioxidant defense against copious generation of ROS. However, the adaptation seems to be ineffective.

Glucose 6 phosphate Dehydrogenase (G6PD)

In the present investigation, the activity of G6PD was observed to be significantly decreased in diabetic patients as compared to controls. Hyperglycaemia hampers G6PD activity and this is supported by earlier findings. It has been reported that at elevated glucose levels, the culture of bovine endothelial aortic cells induced activation of protein kinase A which led to G6PD phosphorylation and hence to a decline in its activity. Similar results were observed in kidney cortex of diabetic rats which were reversed when treated with insulin (Xu et al., 2005). Depressed gene expression and post translational mechanisms both seem to play an intricate role in decreasing G6PD activity which is observed after hyperglycaemic exposure. It has also been observed that elevated glucose diminished the expression of G6PD and its activity in human pancreatic islet cells (Zhang et al., 2010). Hyperglycemia activate adenylate cyclase which in turn promotes the rise in cAMP levels. cAMP on the other hand activates protein kinase A, which is an inhibitor of G6PD. G6PD is the primary source of NADPH, the chief intracellular reductant, hence a decrease in its activity promotes oxidative stress. This was obvious in the study results and it is corroborated by findings of previous studies (Mahmoud and Nor El-Din, 2013).

Catalase

Low Catalase activity was observed in the diabetic subjects of the study as compared to controls. This can be attributed to deficiency of G6PD or to other unknown factors and it may harm heme proteins, result in cell death and along with redox active metal ions may generate highly deleterious hydroxyl radicals (G´oth and Bigler, 2007; G´oth et al., 2005).

Aldose Reductase (AR)

The enhanced activity of AR observed in diabetic patients of the study as compared to controls also added to the existing chaos. It has been reported that activation of polyol pathway also contributes significantly towards oxidative stress (Chung et al., 2003). Over activity of AR exhausts NADPH, which is vital for regenerating reduced glutathione via GR. AR and GR both are potent competitors for NADPH and its inadequate quantity adversely affects the cellular antioxidant capacity (Giugliano et al., 1996). NADPH is a critical product of Hexose MonoPhosphate (HMP) pathway in which G6PD is the rate limiting enzyme. Hence decreased G6PD activity may further lead to depleted levels of NADPH. This NADPH is now the prime target for both AR and GR. AR uses it ultimately to generate superoxide ion (a pro oxidant effect). However, GR on the other hand competes for it to restore glutathione to its stable reduced form (an antioxidant action). Thus the prevailing scenario was that, the superoxide ions generated by AR were still being quenched by SOD owing to its high activity but diminished Catalase and GPx activities were on the verge of tipping the balance in favour of oxidative stress which could lead to diabetic co-morbidities in future.

5.3 Trace metal Analysis

Copper (Cu)

Mean serum copper levels were increased in the study patients as compared to controls. Similar findings have been reported previously (Walter et al., 1991; Zargar et al., 2002). Plasma copper is mostly transported bound to ceruloplasmin (>95%), remaining ions get bound to albumin, transcuprein and copper amino acid complexes. Being an acute phase reactant, Ceruloplasmin shows pro-oxidant activity towards ferrous ion instigated lipid peroxidation and generation of hydroxyl radical in Fenton reaction (Cunningham et al., 1995). Copper is toxic in its free or unbound form and causes redox imbalance owing to its overtly active redox nature, which activates stress sensitive intracellular signalling mechanisms via Haber-Weiss reaction (Cunningham et al., 1995; Mateo et al., 1978). Thus, anomalous copper values might be attributed to hyperglycemia, promoting glycation, thereby stimulating the generation of reactive oxidants that bring about tissue damage (Singal et al., 1999; Zelko et al., 2002).

Zinc (Zn)

In this study, zinc levels in diabetics were lower than the control group. This was in agreement with earlier findings (Pai and Prasad, 1988; Schleinger et al., 1988) but was contradictory with some others (D'Ocon et al., 1987; Mateo et al., 1978; Osman et al., 2004). Zn is crucial for the proper the proper processing, storage, secretion as well as action of insulin in pancreatic beta cells. Hyperglycemia in diabetes causes elevated urinary excretion of Zn ions which causes depletion of Zn ions in the body (Brandao-Neto et al., 2001; Kinlaw et al., 1983). Zn possesses antioxidant properties and can prevent macromolecules from free radical induced oxidation (Singh et al., 1998). Its concentration regulates the metabolism of some other components of antioxidant defense system for example vitamin A and E (Goode et al., 1991; Oberley, 1988;

Tolonen, 1990). Moreover, Zn deficiency has been known to produce high susceptibility to lipoprotein oxidation in experimental models (Disilvestro, 2000).

Thus, in the present study antagonistic relationship was observed in the levels of Cu and Zn ions among the diabetics altering the Cu/Zn ratio which might affect the activity of SOD in future.

Iron

The iron concentration values of T2DM patients with good glycaemic control were statistically non-significant when compared with controls. On the contrary, the free iron concentration values of T2DM patients with poor glycaemic control were significantly higher, both when compared to controls and patients with good glycaemic control. This increase in iron levels may be explained in two different ways. Firstly, iron stores in the pancreas may lead to defective synthesis and secretion of insulin (DeFronzo, 1988). Secondly, excess iron deposition culminates in hyperinsulinemia due to obstruction in the insulin withdrawing ability of the liver (Forouhi et al., 2007). Such deposits hinder insulin action, resulting in insulin resistance, which suppresses the yield of glucose in the liver (Wlazlo et al., 2015). A similar trend has been observed in previous studies (Atari-Hajipirloo et al., 2016; Dulal et al., 2014; Gohel et al., 2013; Perumal et al., 2016; Sudhakar et al., 2014). Poor glycaemic control is the root cause of escalated proteinglycation especially haemoglobin, which restores the free state of iron. This amplified free iron pool revitalizes oxidant generation, conferring damage to biomolecules and leading to complications (Opara, 2004). Elevated transferrin saturation in the diabetic subjects of our study hints at ineffective erythropoiesis and accumulation of iron in human tissues, which hampers insulin action (Fernandez et al., 2002).

As demonstrated in three independent studies, transferrin saturation can act as an independent risk marker for any form of diabetes mellitus, and a value >50% increases the risk of developing T2DM by two to three times (Ellervik et al., 2011). Another study has demonstrated the presence of three to four times higher values of transferrin saturation (>35%) in T2DM patients, compared to the documented values in the general population (Thomas et al., 2004). Such findings have linked elevated transferrin saturation in T2DM patients with earlier age of onset, and our results reflect the same.

Linear relationships between free iron and glycated haemoglobin have been shown in in vitro experiments (Kar and Chakraborti, 1999). It has been shown that H2O2 invokes the release of iron, far more from glycosylated haemoglobin than that from the non-glycosylated form. Similarly, arachidonic acid and deoxyribose in the presence of H2O2 are degraded in a far better way by HbA1c than by non-glycated haemoglobin (HbA0), giving reason to believe that iron release is stupendous with HbA1c as compared to HbA0. On the contrary, the peroxidase activity of HbA1c is less than that of HbA0. Such reactions involving haemoglobin point towards a system of the copious generation of free radicals and oxidative stress in T2DM (Kar and Chakraborti, 2001). The results of our study showed that serum iron concentration in T2DM patients with poor glycaemic control was significantly elevated compared to controls, but it did not show a significant correlation with Tsat or HbA1c values of the same patients. The absence of long-standing diabetic co-morbidities in our T2DM patients may be the reason behind this. When present, these play a crucial role in the vicious cycle of hyperglycaemia and subsequent metabolic distortion (Bozzini et al., 2005; Chandalia and Krisnaswamy, 2002; Shetty et al., 2008). Such variation has also been observed in a survey-based study where the level of serum ferritin (index for body iron stores) showed no correlation with blood sugar and HbA1c in diabetic patients (Sazandeh, 2004).

Elevated serum iron concentration among the general population is found in cases of haemolytic anaemia, hepatitis, and lead and iron poisoning, whereas low serum iron concentration is a marked feature of anaemia caused by iron deficiency due to the impaired intake or absorption of iron, heavy blood loss, late pregnancy, and cancer. The role of iron in the pathogenesis of T2DM calls for further studies owing to increased incidence of iron overload encountered among diabetics, which can be reversed by achieving targets of good glycaemic control using either phlebotomy or iron chelation therapy (Swaminathan et al, 2007).

An increase in the levels of serum free iron concentration and serum transferrin saturation levels with poor glycaemic control in our study indicate an important role of free iron in the development of diabetic complications. A study in Iran has pointed out that elevated levels of iron in first-degree relatives of T2DM patients might be a predisposing factor for them towards the development of diabetes in future or vice versa (i.e., as a result of diabetes development) (Atari-Hajipirloo, 2016). Knowledge and awareness about diabetic complications among the affected individuals and their family is the need of the hour to postpone their onset and progression. Thus, monitoring the prevalence of iron overload is beneficial in the long run.

5.4 Polymorphism Studies

MTHFR A1298C and TGF beta 1 T869C SNP and T2DM complications

Our data displayed no association of MTHFR A1298C SNP for risk of development of T2DM in each of the models examined. Similar trend has been reported in other population (Chehadeh et al., 2016).However, it is important to note that we have not tested for SNP C677T, other SNP for MTHFR, in our subjects. Genotyping both SNPs together could provide further insight for the risk of development of T2DM complications. Also, it will be interesting to examine the level of homocysteine, in

T2DM patients, as these SNPs are known to decrease enzymatic activity of MTHFR. In compromised MTHFR conditions, folate metabolism is expected to be perturbed leading to elevation in blood homocysteine level.

Further, unlike MTHFR A1298C SNP, TGF beta 1 T869C SNP displayed significant association for risk of development of T2DM in dominant and co-dominant models. Risk was highest in dominant model (OR, 3.62) followed by co-dominant model-II (OR, 3.46). Together, it displayed that heterozygous TC genotype has more risk to develop T2DM. The fact is well reflected in genotypic distribution of TC in control and T2DM subjects. Thus, frequency of TC heterozygotes was higher in T2DM subjects (51.67%) in comparison to controls (26.67%). Similar trend for association of SNP T869C for risk of T2DM development has been reported by others in different populations (for details please refer to section 2.10.1). As SNP T869C is associated with more secretion of TGF beta1, serum concentration of TGF beta1 is expected to be higher in T2DM subjects. Also, our study suggests that TC heterozygotes should be alerted, particularly if they are predisposed for T2DM. For instance, a heterozygous carrier with T2DM in first degree of blood relatives should take necessary steps to safeguard him/her from potential T2DM complications.

PTPN22 C1858T SNP

Although there have been reports of association of PTPN 22 C1858T SNP in other populations (Douroudes et al., 2008, Cervin et al., 2008) but it was found to be non-polymorphic in our study population. This is corroborated by the fact that TT genotype and T1858 allele has not been shown to be present in Asian, African and American populations. Moreover, T allele has reported to be virtually absent in Chinese, Japanese and Korean populations (Vang et al., 2007). Thus, ethnic variations owing to founder effect may be the reason behind the study results.

Metabolomic (NMR) analysis

As expected glucose levels were upregulated in the diabetic patients (both with and without complications) as compared to controls. Similar trend has been reported before (Li et al., 2009; Suhre et al., 2010). Metabolites which were shown to be down regulated in diabetic patients with complications (DC) as compared to those patients without complications (DB) and normal controls (NC) included branched chain amino acids (BCCA) valine and isoleucine. An earlier study analysed the plasma of T2DM patients who eventually developed end stage renal disease (ESRD) within 8-12 years of follow up. Their results also showed significant down regulation of BCCA, and aromatic amino acids. This could be due to superfluous mitochondrial amino acid ß oxidation (Niewczas et al., 2014). Another study compared metabolites of T2DM patients with and without nephropathy and reported depleted levels of BCCA and aromatic acids (Huang et al., 2013). Hence, these results upon further analysis could be used to prevent ESRD in the diabetic patients of our study.

One of the metabolites that was found to be down regulated in the patients (DC) as compared to DM and NC was glutamate. Similar results have been reported in previous studies (Messana et al., 1998; Newgard et al., 2009; Lanza et al., 2010). This finding was especially relevant in the context of this study as it corroborated with MTHFR A1298C polymorphism that involves conversion of glutamate to alanine. Similarly, proline was found to be down regulated in the patients with complications (DC) as compared to those without (DB) and normal controls (NC). TGF ß T869C polymorphism that causes mutation of leucine to proline is implicated in diabetic nephropathy. This polymorphism was found to be highly significant in the study patients as compared to controls. When the samples for biochemical and polymorphism studies were procured from the patients, complications were not present in them. But at

the time of sampling for NMR analysis, the patients (from the original set of patients) had developed complications (DC) including nephropathy and were undergoing treatment for them. Hence, the down regulation of proline seemed to be the effect of ongoing medication. Up regulation of urea also points towards decline in kidney function.

With respect to metabolites of tri carboxylic acid (TCA) cycle, patients had elevated citrate levels. This has been reported in a previous study which found that these levels were also associated with glycosuria (Messana et al., 1998). Based on our biochemical and genetic polymorphism trends we argued that the condition of the patients might get worse leading them towards diabetic co-morbidities in near future. This prompted us to conduct this follow up study using NMR metabolomics and the outcomes were in agreement with our earlier predictions. Thus NMR can be employed as a predictive tool which would aid the routine treatment modalities in benefitting the patients.

5.5 Awareness and adherence profile of patients with type 2diabetes

The main motivation behind this questionnaire based study was to obtain a snapshot of the awareness profile and to tap the erroneous zones that lead to non-adherence by the patients, leading to poor treatment outcome.

The principal finding of the study was that only 52.7% of the patients knew that they suffered from type 2 diabetes. CURES-9 study in Chennai had pointed out about one third of the general population was ignorant about diabetes (Mohan et al., 2005). A study in Bangladesh reported that the knowledge level of old diagnosed patients who had been educated on diabetes was the same as those of newly diagnosed diabetics or non-diabetic subjects (Saleh et al., 2016). In ICMR-INDIAB study it was reported that among diabetic population 63.4% knew that the disease is preventable. However, in this study population, only 53.93% subjects were aware of this fact. This difference

confirms the finding of ICMR-INDIAB study that sharp rural-urban gradient concerning diabetes awareness is evident in north India as against uniform awareness profile in south India. 69% of our study subjects replied affirmatively in favour of obesity being a diabetes risk factor. Our results match that of a Bangladesh study where 71% study participants recognized that being overweight was a risk factor for diabetes (Mumu et al., 2014). Sedentary lifestyle was recognized as a risk factor by 61.2% of our study subjects.

An awareness study conducted at Kolkata pointed out that the participating diabetic patients were alive to only a few features of the disease pertaining to prevention, control, symptoms and complications (Mukhopadhyay et al., 2010). Only 34.4% of their patients knew about the family history aspect of the disease as against 50.3% patients in our study. Moreover, 67.15% participants of Kolkata study confessed of getting information about diabetes from their healthcare provider. The results of this study corroborate the same with 67.87% of the study patients giving credit to their attending physician for providing knowledge about diabetes. Other studies have also highlighted the role of healthcare staff as the primary information giver (Al-Mahrooqi et al., 2013).

Heart problem was the most commonly recognized diabetic complication by the patients. This is corroborated by other studies where majority of diabetic population emphasized heart disease to be the most disturbing diabetic co-morbidity (O'Sullivan et al., 2009). Another study conducted across 11 cities and 9 states in India found kidney and eye diseases to be the most commonly reported diabetic co-morbidities (Gudlavalleti et al., 2016). Thus, imparting knowledge about diabetic complications may motivate newly diagnosed patients to adopt preventive lifestyle measures.

Diabetes is a costly affair to deal with. This is evident from this study results where 73.33% of participants admitted facing economic burden in continuation of their

treatment. Cost of routine tests and medication has been reported as a challenging aspect of diabetes treatment (Gudlavalleti et al., 2016). Moreover, a significant number of participants in a Delhi based study acknowledged that treatment cost was a deterrent to prolongation of their therapy (Kishore et al., 2015). It has been estimated that, in our country, on an average, a mere 10-12% of diabetic patients are able to obtain latest pharmacological treatment (Kapur et al., 1997). Most of the diabetic subjects in India rely on their family support in combating this illness. So, if an earning member of the family develops diabetes, it hits hard on the everyday resources of the family and leads to long lasting financial consequences (Kapur, 2007). Furthermore, there is a wide disparity observed between the treatment facilities available at private and government centres. Major recommended guidelines for diabetes related care are not being followed in most government run health care centres (Tharkar et al., 2011). Counselling pertaining to healthy and preventive lifestyle, dietary regimen and physical exercise was administered at some specialized diabetes centres only and was more or less negligible at government run centres (Tharkar et al., 2011). The study patients witnessed similar scenario at their respective treatment centres.

Patient's realizations of the existing health care management system are generally sidelined by the authorities in developing country like ours. Patient gratification is a tangled web of intricately woven factors like previous experiences, future surmises, life style patterns, efficacy of available clinical services, quality of interaction with physician and medical staff, physical ease and importance to patient's priorities (Jenkinson et al., 2002). In our study, 62.4% participants were satisfied with the quality of patient –physician interaction. However, another study at Bareilly U.P., reported that patients were more content with the behaviour of staff members (IIIrd and IVth class employee) than that of doctors and nurses (Singh et al., 2014). Thus, patient-physician

interaction is vital in spreading knowledge about medication and other treatment modalities which directly affects adherence (Martin et al., 2005). It has also been suggested that dialogue between patient and healthcare staff may help in reconciling patient's distress over the disease and is shown to have positive correlation with adherence profile of the patient (Rubin et al., 2006). 36.96% of the study participants confessed that they feel stressed out at their diseased status. A study from north India has reported that up to 41 % of diabetic participants suffered from depression (Raval et al., 2010). There is very limited data on co-occurrence of diabetes and depression. The recognition and addressal of their association can have lasting impact on the prevention and treatment of both. However, various factors like limited awareness on guidelines, time constraints, facility crunch, focus on short term symptomatic relief rather than opting for preventive care and delayed action on poor control retard the proper management of disease (Mohan et al., 2009). Another study has pointed out that nearly 92.3% of diabetic patients availed the services of a general practitioner for therapy instead of a specialist diabetologist (Murugesan et al., 2007). Most of the study subjects who sought treatment at government hospitals did the same as treatment cost was much cheaper than that of private clinics. This slipshod attitude of people can only be corrected when they are made aware of the full implications of the disease which is only possible through massive knowledge and awareness campaigns. Health care resources available in India and other developing nations are few with only 5% GDP (Gross Domestic Product) getting spent on healthcare programmes (Peters et al., 2002). Thus meticulous planning is the need of hour for rational use of funds for prevention and treatment of diabetes (Kapur, 2007).

Non-adherence among diabetics may also be attributed to adverse side effects of prescribed medications. Popular therapeutic options are related with hypoglycaemia and

weight gain which has led to low or moderate compliance in patients (Dilla et al., 2008). In a survey study among type 2 diabetics in USA, 71.7% complained of facing at least one of the side effects (hypoglycaemia, weight gain, constipation/diarrhoea, water retention and headaches) in the first two weeks. Each of the side effects was attached with 28% higher probability of non-adherence (Pollack et al., 2010). About 30.9% of this study subjects complained of facing one or the other above mentioned issues which further led some of them (13.9%) to skip medicine when they felt their symptoms were under control. Moreover, 28.48% confirmed that they sometimes forgot to take their medicines. This calls for attention to all those concerned as the situation is alarming. Patient's connection with his/her therapy is one of the most crucial factors for successful implementation of the same and requires stimulus, knowledge and acceptance to a demanding and tortuous lifelong regimen. Effective T2DM tutelage with enhancements in understanding, perspectives and expertise about the disease results in its superior regulation and is pivotal to T2DM management and care (Asha et al., 2004;Norris et al.,2002). Lack of participation in disease treatment by patient as well as other community members leads to poor patient outcome and is the major stumbling block in handling the disease successfully (Jing et al, 2008). Self-motivation and management training is the key to tackling diabetes in the long run as it requires the patient to be acquainted with the inherent nature of disease, associated risk factors, treatment modalities and possible co-morbidities. Importance of in depth education in diabetes management has been highlighted by many studies (Brown, 1990; Tan et al., 1997). Exhaustive diabetes management and education can ameliorate glycaemic control, patient's outcome as well as quality of life (McMurray et al., 2002).

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APPENDIX

Health Assessment Questionnaire

1. Name……. Patient ID…….

2. Sex …… Age …….

3. BMI …… Disease Duration ……

4. Family History of diabetes (a) Yes …… (b) No ……

5. Marital Status (a) Single…… (b) Married…….. (c) Widowed……..

6. Education (a) Junior H.S. …….. (b) Intermediate……. (c) Graduate…….(d) University…….

7. Occupation (a) Service……. (b) Business ……..(c) Unemployed…… (d) Others …….

8. Economic Status (a) LIG…….. (b) MIG …… (c) HIG ……..

9. Regular Follow –up visit to the clinic (a) Yes…… (b) No ……..

10. Regular use of prescribed drugs (a) Yes……..... (b) No ……....

11. Self testing of blood glucose at home (a) Yes…… (b) No …….....

12. Compliance with diet regulations (a) Yes…… (b) No ……..

13. Source of information about disease ……..

14. Knowledge about diabetes symptoms…….

14. Knowledge about future diabetic complications ……

15. Do you know what type of diabetes you have? …….

16. Do you know about the cause of diabetes? .....

17. Do you know that it can be prevented and controlled? .....

18. Knowledge abour risk factors……

19. Need help in reaching clinic (a) Yes …… (b) No …….

20. Positive role of family in disease care and management (a) Yes …… (b) No ……

21. Smoking (a) Yes …… (b) No …….....

22. Drinking (a) Yes …… (b) No……..

23. Exercise (a) Mild …….. (b) Moderate …….. (c) Vigorous……..

24. Cooking Medium (a) Mustard oil …….. (b) Refined oil ……(c) Dalda ……

25. Consumption of junk food (a) rarely ……. (b) Sometimes ……..(c) Frequently……..

26 Are you forgetful about your medicines sometimes? (a) Yes……..(b) No……..

27. Do you experience ant side effects with the prescribed medicines? (a) Yes…….(b) No……..

28. Do you sometimes skip medicines when you feel your symptoms are under control?

(a) Yes…….(b)No….....

29. Do you feel stressed out at your disease status? (a) Yes……..(b) No…….

30. Are you dissatisfied with your interaction with the physician? (a) Yes…….(b) No……

31. Do you face economic crunch in continuation of your treatment? (a) Yes…….(b) No……

32. Do you follow any alternative form of treatment (homeopathy, ayurvedic etc.)? (a)Yes…….(b) No……..

List of Publications

- G Misra, SK Bhatter, A Kumar, V Gupta and MY Khan. Iron Profile and Glycaemic Control in Patients with Type 2 Diabetes Mellitus. Medical Sciences 4,22 (2016).
- G Misra, SK Bhatter, A Kumar, V Gupta and MY Khan. Prevalence of Comorbidities in patients with Type 2 Diabetes mellitus. Int. J. Adv. Res. 4(11), 2016 1881-1890.
- G Misra, V Gupta and MY Khan. Prevalence of Metabolic Syndrome in Type 2 Diabetic Patients in Kanpur region of North India (Under review in Journal of Diabetes Mellitus)

Manuscript under preparation

- Awareness and adherence profile of patients with type 2 diabetes in north India
- Pathophysiology of Type 2 Diabetes- Dilemma of today's times.

medical sciences

MDPI

Article

Iron profile and glycaemic control in patients of Type 2 Diabetes Mellitus

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Abstract: Iron overload is increasingly being connected to insulin resistance in T2DM patients. Free iron causes production of reactive oxygen species (ROS) which may lead to oxidative stress mediated diabetic complications. The study aims to access the serum iron, total iron binding capacity (TIBC) and percentage transferrin saturation of 150 subjects out of them patients with type 2 DM with good glycemic control were 50(Group II), patients with type 2 DM with poor glycemic control were 50 (Group III) and 50 normal healthy control (Group-I). Mean serum free iron concentration was 105.34±3.5, 107.33±3.45 and 125.58±3.45 μg/dl in in Group-I, Group II and Group III respectively. Mean serum TIBC concentration in Group-I, Group II and Group III was 311.39±5.47, 309.63±6.1 and284.2±3.18μg/dl respectively. Mean serum Transferrin saturation (%) in Group-I, Group II and Group III was 34.17±1.21, 35.02±1.2 and 44.39±1.07 respectively. The difference between TIBC, mean serum free iron concentration and Transferrin saturation between group I and group III (for all, p values <0.0001) as well as between group II and group III (p value 0.0005, 0.0004 and<0.0001 respectively) is statistically significant. No significant correlation was observed between serum iron and HbA1c (r= 0.05) and Transferrin sauration (r= 0.0496) in group III.

Keywords: T2D;, Glycaemic control; Serum Iron; Transferrin saturation

PACS: J0101

1. Introduction

The gigantic leap in diabetic population is a serious health concern worldwide. Chronic hyperglycemia along with derangement of carbohydrate, fat and protein metabolism is its characteristic feature resulting from aberrations in insulin secretion, insulin action or both.

Mineral elements are not only structural components of body tissues but are also inherently involved in various physiological processes of metabolism. Their role is well documented in the production, storage and release of insulin as well as its conformational integrity [1]. Insulin resistance is synonymous with diabetes and various studies point to its connection with iron overload in body. Iron is a transition metal capable of redox activity and any potential harm targeted at the body is prevented by its binding with transport or storage proteins [2]. In blood iron is carried by transferrin, a protein made in liver. In its free form i.e., in non-transferrin bound form

it is known to induce oxidation of biomolecules through Haber-Weiss and Fenton reactions by producing harmful hydroxyl radicals [3]. These free radicals are powerful prooxidants which cause lysis of lipid cellular membrane, damage protein structural configuration and displace nucleic acid in genes [4,5]. Thus the catalytic action of free iron contributes initially to insulin resistance and later on to reduced insulin release which subsequently results in the development of T2DM [6-8]. Emerging scientific evidence has disclosed that the relationship is bidirectional wherein glucose metabolism also impinges upon several iron metabolic pathways. Inflammatory cytokines in oxidative stress influence these relationships, replicating and intensifying the initiated events. Chronic diabetic complications are also modulated by iron induced damage [9].

Variable findings have been reported for iron profile status in different studies and hence the present study aims to determine the status of parameters related to iron metabolism in the selected population.

2. Materials and Methods

The study was designed as a case control study of diabetic patients visiting the outpatients department of government hospitals and some private clinics in Kanpur city. The study consists of 150 subjects out of them 50 patients having type 2 diabetes mellitus with good glycemic control (Group II), 50 patients with type 2 diabetes mellitus with poor glycemic control (Group III) and 50 normal healthy control (Group-I) were selected. The selection was random to minimize any bias.

Inclusion Criteria:

The subjects selected for study were grouped as follows:

Group I – Control group (n=50), consists of age and sex matched healthy subjects. They were free from any ailment which could affect the parameters under study. They were not on any medication. They were taken from general population.

Group II – Diabetes Mellitus type 2 patients with good glycaemic control (n=50), disease duration less than 7 years and glycated hemoglobin (HbA1C) level less than 8%. They were on life style modifications and oral hypoglycaemic drugs.

Group III – Diabetes Mellitus type 2 patients with poor glycaemic control (n=50), disease duration more than 7 years and Glycated hemoglobin (HbA1C) level more than 8%. They were on life style modifications, oral hypoglycaemic drugs, insulin or combination of all three.

Exclusion criteria:

The exclusion criteria for patients extended to those diagnosed with type 1 DM, acute complications such as severe infections or major operations, trauma, severe cardiovascular/respiratory diseases, pregnant and breast feeding women. Newly diagnosed cases and those suffering from chronic diabetic co-morbidities were also excluded from the study.

The study was approved by institutional ethical committee and written informed consent was obtained from all the study subjects after explaining the objectives of the study. All possible precautionary measures were taken to prevent trace metal ion contamination particularly iron, during all stages of the procedure. All spectrometric readings were done using UV-1800 SHIMADZU spectrophotometer.

Fasting venous blood was collected using standard clinical procedures. Iron and TIBC were estimated by using commercial kit (Ferrozine method) produced by Coral Clinical systems. Iron bound to transferrin, is released in an acidic medium and the ferric ions are reduced to ferrous ions.

The Fe (II) ions react with ferrozine to form a violet coloured complex. Intensity of the complex formed is directly proportional to the amount of iron present in the sample. For TIBC, the serum is treated with excess of Fe (II) to saturate the iron binding sites on transferrin. The excess Fe (II) is adsorbed and precipitated and the iron content in the supernatant is measured to give the TIBC. Transferrin saturation TSAT (in %) is the ratio of serum iron and TIBC, which tells how much serum iron is actually bound.

Transferrin saturation (%) = serum iron x 100/ serum TIBC

Statistical analysis-All the values are expressed as Mean±SEM and p value of <0.05 was considered statistically significant. Statistical analysis was done using Graph Pad prism 6 software. Significance in differences between mean values was assessed from unpaired t test. Correlations of serum iron with other parameters were also studied by applying Pearson correlation test. Results were interpreted on the basis of comparison between controls and diabetic subjects.

3. Results

Table1 shows the general features of healthy controls and cases.

Table 1: General Features of healthy controls and cases

Parameter	Group I	Group II	Group III
Number	50	50	50
Disease duration (years)	-	<7	>7
HbA1c(%)*	6±1	7±1	>8

*data not shown

Present study was undertaken to assess level of serum free iron, serum TIBC and Tsat concentration in type 2 diabetes mellitus patients with good and poor glycaemic control and compare it with healthy controls. As shown in table 2, 3 and 4 mean serum free iron concentration in Group-I, Group II and Group III was 105.34±3.5, 107.33±3.45 and 125.58±3.45 µg/dl respectively while mean serum TIBC concentration in Group-I, Group II and Group III was 311.39±5.47, 309.63±6.1 and284.2±3.18µg/dl respectively.

Table 2: Comparison of Iron profile between Group I (Healthy controls) and Group II cases (T2DM with good glycaemic control)*

Parameter	Group I	Group II	P value
Serum iron (µg/dl)	105.34±3.5	107.33±3.45	0.686
TIBC (µg/dl)	311.39±5.47	309.63±6.1	0.829
Tsat(%)	34.17±1.2	35.02±1.2	0.622

**Values expressed in mean±SEM*

Table 3: Comparison of Iron profile between Group II (T2DM cases with good glycaemic control) and Group III (T2DM cases with poor glycaemic control)*

Parameter	Group II	Group III	P value
Serum iron (µg/dl)	107.33±3.45	125.58±3.74	0.0005
TIBC(µg/dl)	309.63±6.1	284.2±3.18	0.0004
Tsat(%)	35.02±1.2	44.39±1.07	<0.0001

**Values expressed in mean±SEM*

Table 4: Comparison of Iron profile between Group I (healthy controls) and Group III (T2DM cases with poor glycaemic control)*

Parameter	Group I	Group III	P value
Serum iron (µg/dl)	105.34±3.5	125.58±3.74	**0.0001**
TIBC(µg/dl)	311.39±5.47	284.2±3.18	**<0.0001**
Tsat(%)	34.17±1.2	44.39±1.07	**<0.0001**

**Values expressed in mean±SEM*

Further, as displayed in table 2, 3 and 4 mean serum Transferrin saturation (%) in Group-I, Group II and Group III was 34.17±1.21, 35.02±1.2 and44.39±1.07µg/dl respectively. The difference between mean serum free iron concentration, TIBC and Tsat between group 1 and group 3 (all three p values <0.0001) as well as between group 2 and group 3 (p value 0.0005, 0.0004 and<0.0001 respectively) is statistically highly significant. But the difference between these parameters in group 1 and group 2 is not significant (p value is 0.6868, 0.8298 and 0.6221 respectively).

Statistical analysis showed that there was no significant correlation between serum iron and HbA1c (r= 0.05) and Tsat (r= 0.0496) in diabetic patients of group III (table 5).

Table 5: Corelation of serum iron concentration with HbA1c and Tsat in group III cases

Parameter	Pearson coefficient (r)	P value
HbA1c	0.05	0.73
Tsat	0.0496	0.732

4. Discussion

In our study serum iron was found to be slightly high, TIBC slightly low and percentage transferrin saturation slightly high in diabetes patients (both group II and III) as compared to healthy controls (group I). This increase in iron levels may be explained in two different ways. Firstly, iron stores in pancreas may lead to defective synthesis and secretion of insulin [10]. Secondly, excess iron deposition could interfere with insulin extracting capacity of liver resulting in hyperinsulinemia [11]. Iron deposits in liver may lead to insulin resistance by interfering with the action of insulin which suppresses hepatic glucose production. However this increase in iron concentration values of group II was statistically non-significant when compared with controls (group I). On the contrary increase in free iron concentration values of group III was statistically significant both when compared to group I as well as group II. Similar trend has been observed in previous studies [12,13]. Poor glycaemic control causes increased glycation of proteins, especially hemoglobin, which releases the iron in its free state. This increased presence of free iron pool revitalizes oxidant generation conferring damage to biomolecules and leads to complications.

Elevated transferrin saturation in diabetic subjects of our study hints at ineffective erythropoiesis, accumulation of iron in human tissues which hampers insulin action [14]. As demonstrated in three independent studies, transferrin saturation can act as an independent risk marker for any form of diabetes mellitus and a value≥50% was associated with two to three fold increased risk of developing T2DM [15]. Another study has shown that the prevalence of elevated transferrin saturation (> 35%) was 3–4- fold higher in patients with diabetes mellitus, compared with historical prevalence in the general population [16]. These studies have associated elevated transferrin saturation in T2DM patients with earlier age of onset which is also true of our findings.

Earlier studies have proved that poor glycemic control enhances protein glycation especially of haemoglobin which releases iron in its free state [14]. Linear relationships between free iron and glycated haemoglobin have been shown in in-vitro experiments [17]. Effect of glycosylation on iron-mediated free radical reactions of hemoglobin has shown that H_2O_2 induced iron release is more from HbA1c than that from non glycosylated hemoglobin (HbA0). In the presence of H_2O_2,

HbA1c degrades arachidonic acid and deoxyribose more efficiently than HbA0, which suggests that iron release is more with HbA1c compared to HbA0. HbA1c exhibits less peroxidase activity than HbA0. These findings on glycosylation-induced functional properties of hemoglobin suggest a mechanism of increased formation of free radicals and oxidative stress in diabetes mellitus [18]. The results of our study showed that serum iron in diabetic patients with poor glycemic control (group III) is significantly higher than controls, but its level has no significant correlation with Tsat or HbA1c in patients of group III. This difference can be due to our exclusion criteria such as severe diabetic complications that play crucial role in the vicious cycle of hyperglycemia and subsequent metabolic distortion. Such variation has also been observed in a survey based study where the level of serum ferritin (index for body iron stores) showed no correlation with blood sugar and HbA1c in diabetic patients [19].

Increased serum iron levels among general poulation are found in hemolytic anemias, hepatitis, lead and iron poisoning whereas decreased serum levels are found in anemias caused by iron deficiency due to insufficient intake or absorption of iron, chronic blood loss , late pregnancy and cancer. The role of iron in the pathogenesis of T2DM calls for further studies owing to increased incidence of iron overload encountered among diabetics which can be reversed by achieving targets of good glycaemic control using either phlebotomy or iron chelation therapy [20].

Increase levels of serum free iron concentration and serum transferrin saturation levels with poor glycaemic control in our study indicate important role of free iron in development of diabetic complication. Thus the study of iron and related parameters can be a useful offshoot of the conventional studies on diabetes and its complications. Iron overload associated with poor glycaemic control can be harnessed as a valuable marker for diabetes pathogenesis. Similar studies with larger cohort size including patients with co-morbidities will further present an expanded view of the current situation.

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RESEARCH ARTICLE

PREVELANCE OF COMORBIDITIES IN PATIENTS WITH TYPE 2 DIABETES MELLITUS.

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Abstract

Type2 Diabetes affects a huge population of the world with India on the top of the world's list. Present study aims to gain information about the status of diabetes in the study population laying emphasis on the prevalence of co-morbidities. The study was done on 120 asymptomatic controls and 120 diabetics. A standardized questionnaire was used to collect socio-economic and demographic details of the participants. The mean duration of diabetes was 7.07±0.5years. The mean BMI values among diabetics and controls were 27.25±0.25 kg/m^2 and 25.367±0.263 kg/m^2 respectively (p<0.001). About 24.16% patients had abnormal ECG values while 75.83% patients showed typical dyslipidemia. 60% of the affected individuals had abnormal HbA1c values (> 7) and 17.5% patients had frequent non-healing skin and soft tissue infections or ulcers of leg or foot. Kidney function tests displayed deranged values in about 11.66% patients. 62.5% diabetics belonged to the age group of 46-60 (p<0.0018) and 67.5% of them had positive family history (p<0.0001). Socio-demographic factors like marriage, education, occupation, smoking, exercise, cooking medium and consumption of junk food were found to be significantly associated with the disease. Effective treatment modalities and awareness is necessary for diabetes management in known cases and for early diagnosis in future cases.

Introduction:-

Diabetes is an enigma, genetically inherited, very essentially a metabolic disease that is revealing its secrets slowly to the researchers all over the world. There has been a quick upswing in the number of diabetic patients and this stupendous growth is noted in urban as well as rural areas. Ever increasing industrialization, narrowing urban-rural divide, amplified economic growth, changing dietary norms, lesser or no physical activity and alleviated stress levels among all strata of society are the risk factors behind the devil called Diabetes.

In India, the disease is scaling the heights of becoming a potential epidemic with more than 62 million individuals currently diagnosed with it (Joshi and Parikh, 2007; Kumar et al., 2013). The prevalence of diabetes is predicted to

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double globally from 171 million in 2000 to 366 million in 2030 with a maximum increase in India (Wild et al., 2004). It is predicted that by 2030, Diabetes mellitus may afflict up to 79.4 million individuals in India while China (42.3 million) and United States (30.3 million) will also face a significant upsurge in the number of affected individuals (Wild et al., 2004; Whiting et al., 2011). From being considered as the disease of the elderly, diabetes has now become the major cause of morbidity and mortality affecting youth and middle aged as well. Increased prevalence is responsible for putting socio-economic pressure on the most productive age group and health systems in the country (Mohan et al., 2007). 80% of the total burden of diabetes mellitus is from developing countries of which major contributors are India and China. Indians have a high ethnic and genetic susceptibility for the disease, and also have lower threshold limits for the environmental risk factors. It is a matter of major concern that Indians develop T2DM at a younger age than the western populations (Ramachandran et al., 2010).

Despite the increasing incidences of diabetes, a very few multi-centric studies are being conducted on the prevalence of diabetes and its complications (Kaveeshwar and Cornwall, 2014). As far as northern India is concerned, a few epidemiological studies have been conducted in Delhi (Ramachandran and Snehalatha, 2009, Ramachandran et al.; 2001; Misra et al, 2001), Chandigarh (Anjana et al., 2011) and Kashmir (Zargar et al., 2000) in the past. But there is dearth of information available about the disease status at Kanpur district. Owing to better and cheaper medical facilities available here people from surrounding districts and cities flock to it for treatment. Our aim in this study was to gain a detailed insight into the prevalence of co-morbidities in diabetic subjects as well to access the socio-economic as well as demographic risk factors related to the disease in both controls and diabetics.

Materials and Methods:-

Study design- The layout of this study was designed in the rheumatology laboratory of CSJM University, Kanpur in Uttar Pradesh, India. The diabetic patients (n=120) were selected randomly from those attending the OPD of GSVM medical college and hospital and some private clinics in Kanpur. To compare the findings an equal number of age-matched healthy individuals were studied. The population studied comprised of mostly literate people of different socio-economic strata of Kanpur and other surrounding districts. The subjects included were mainly professionals, skilled workers, housewives, retired persons, businessmen and teachers and their staple food was mainly wheat.

Definitions:-

Obesity was defined as BMI >25 kg/m2 (World Health O., 2000). Persistent elevation of blood pressure >140/90mmHg was defined as hypertension. Dyslipidemia was defined by the criteria laid down by National Cholesterol Education Program, Adult Treatment Panel II (National Cholesterol Education Panel, 1994).

Methodology:-

Fasting venous blood was collected using standard clinical procedures**.** Blood glucose was estimated by Autospan kit (GOD-POD, end point and kinetic assay method) of Span diagnostics. HbA1Cwas estimated by the modified colorimetric method of *Fluckiger* (Fluckiger, 1976). In-vitro diagnostic kits from Span diagnostics Ltd. were used For Triglycerides estimation (GPO-PAP end point assay), from Beacon diagnostics Pvt. Ltd. For cholesterol estimation (CHOD-POD method) and from Crest Biosystems for HDL-C estimation (PEG precipitation method).VLDL-C and LDL-C was calculated *Friedewald's* and *Fredrichson's* formulae (1972) (Friedewald et al., 1972).

VLDL-C= TG/5 and LDL-C= T.C- (HDL-C + VLDL-C)

Uric acid (uricase/PAP method) and Creatinine values were estimated in serum by kits from Coral clinical systems. All the estimations were done using UV-1800 SHIMADZU UV Spectrophotometer

With the help of a detailed predesigned questionnaire, information was collected on socio-demographic characteristics, family history of diabetes, smoking and drinking habits, the basic know-how about the disease, its complications, care and awareness pattern associated with it and their physical activity levels. Height and weight were recorded by standard methods and BMI was calculated (kg/m^2).The study was approved by the institutional ethics committee. An informed consent was duly obtained from every participant.

Statistical analysis:-
Data were recorded, tabulated and appropriate statistical test was used wherever applicable. A *p* value of less than 0.05 was considered statistically significant. *P* value represents the difference between type 2 diabetic patients and non-diabetic controls. Intable 1, clinical data are presented as mean ± SEM. Group means were compared by unpaired- t test using Graph-pad software (version 6). Data pertaining to table 3, 4 and 6 were assessed by chi-square test. Data of table 2 and 5 were expressed in frequency and percentage.

Results:-

All the 120 diabetic patients screened had complete records of their clinical profile. Table 1 comprises of the general features of the subjects. Male/female ratio in diabetics was 83/37 as compared to 69/51 seen in controls. The duration of disease ranged from 1 to 28 years with a mean of 7.07±0.5 years. Significantly higher BMI values were obtained in diabetic subjects (27.25±0.25) kg/m^2 as compared to controls (25.367±0.263) kg/m^2.

Table 2 summarizes the results of various tests and clinical facilities utilized by the patients on the prescription of their attending physician for tracking the development of co-morbidities. About 24.16% patients had abnormal ECG values while 75.83% patients showed typical dyslipidemia. 60% of the affected individuals had abnormal values of glycated hemoglobin (> 7) and 17.5% patients had frequent non-healing skin and soft tissue infections or ulcers of leg or foot. Kidney function tests displayed deranged values in about 11.66% patients. To validate the current status of these complications corresponding laboratory tests were performed on patients as well as controls and the results shown in table 1. There was a significant increase observed in fasting glucose (180.5±5.46mg/dl *vs* 108.62±1.44mg/dl) and HbA1c (7.89±0.083 *vs* 6.67±0.042) indicating a state of persistent hyperglycemia in diabetics. Similarly significantly higher values of blood pressure (systolic and diastolic) were observed in diabetics as compared to controls. Lipid profile showed significantly higher values in fasting TG (257.36±7.27mg/dl *vs* 139.14±2.74mg/dl), TC (234.07±4.1mg/dl *vs* 183.52±3.29mg/dl), LDL-c (148.61±4.23mg/dl *vs* 115.63±3.43mg/dl) and VLDL-c (51.47±1.45mg/dl *vs* 27.83±0.55mg/dl) whereas HDL-c values were significantly lower in patients (33.98±0.52mg/dl *vs* 40.06±0.61mg/dl). Significantly higherUric acid (5.86±0.16mg/dl *vs* 3.94±0.08mg/dl) and Creatinine values (1.06±0.02mg/dl *vs* 0.99±0.013mg/dl) were also observed.

Data concerning the age-wise distribution of diabetic subjects is shown in table 3. Majority of the patients (62.5%) belonged to the age group of 46-60 followed by >60 age group (20.83%). Chi square analysis showed highly significant association ($p<0.001$) between age distribution pattern and T2DM.

Table 4 shows the family history of the subjects. 67.5% diabetics had a family history indicating the strong genetic component of the disease. However, 14.16% controls also showed family history suggesting their susceptibility for development of the disease. Results of chi square analysis showed extremely significant association ($p<0.0001$) between family history of diabetes and T2DM.

Table 5 depicts the pattern of care and awareness among the diabetics as well as healthy controls. 86.66% patients appeared for regular follow-up visits and about 82.5% were regular in using the prescribed drug regimen however only 75.83% complied with dietary recommendations. Those practicing self-monitoring of glucose at home constituted only 26.6% of the patients. A basic knowledge about the disease was present in about 67.5% of the patients as against 56.66% of the controls whereas knowledge pertaining to future complications was seen to vary from 43.33% in controls to 55% in diabetics. 9.16% patients needed physical help in reaching the clinic. The outstanding feature of the table is the highly positive role of family (94.16%) in administering proper care to the diseased individual.

Table 6 shows the demographic features of the subjects. Diabetic subjects were present in all the three economic strata with highest (38.33%) in MIG followed by HIG (35%) and LIG (26.66%). However the trend was not statistically significant. Servicemen appeared to be the frontrunners in the occupation section (50.83% diabetics) followed by business men (26.66%), unemployed (5%) and others (17.5%). The education profile pointed out that about 40.83% patients were graduates while slightly less than a quarter of them (24.16%) were intermediate qualified, one-fifth (20%) of the patients held university degrees and 15% were junior high school pass outs. Majority of the subjects were married (80.83%) while the rest were single (14.16%) and widowed (5%). Non-smokers (81.66%) and non-drinkers (79.16%) comprised a significant group of the diabetic subjects. The difference between alcohol consuming patients and controls was non-significant. Mild physical activity was the most prevalent form of exercise among the patients (84.16%) followed by moderate and vigorous one. Refined oil was the chosen

cooking medium among the patients (60.83%). 60% of the patients accepted that they sometimes enjoyed junk food while 36.66% accepted to doing so frequently and only a meagre 3.33% did so rarely.

Table 1:- General and clinical *features of subjects (controls and diabetics),* n is the number of participants.

Parameter	Control(n=120)	Diabetic(n=120)	*P* value
Sex(male/female)	69/51	83/37	-
BMI (kg/m^2)	25.367±0.263	27.25±0.25	<0.001
Disease duration(year)	NA	7.07±0.51	-
Plasma glucose (mg/dl)	108.62±1.44*	180.5±5.46*	<0.001
HbA1c(%)	6.67±0.042*	7.89±0.083*	<0.001
Systolic BP (mmHg)	125.79±0.84*	143.24±1.45*	<0.001
Diastolic BP (mmHg)	81.34±0.48*	87.73±1.09*	<0.001
Creatinine (mg/dl)	0.995±0.013*	1.068±.024*	<0.05
Uric acid (mg/dl)	3.94±0.08*	5.86±0.16*	<0.001
TC (mg/dl)	183.52±3.29*	234.07±4.10*	<0.001
TG (mg/dl)	139.14±2.74*	257.36±7.27*	<0.001
HDL (mg/dl)	40.06±0.61*	33.98±0.52*	<0.001
LDL (mg/dl)	115.63±3.43*	148.61±4.23*	<0.001
VLDL (mg/dl)	27.83±0.55*	51.47±1.45*	<0.001

values are expressed as mean ± SEM,

Table 2:- Clinical facilities utilized by the diabetic patients (within one year) for tracking co-morbidities*

Facility	No. of patients availing the facilities (%)	No. of patients with abnormal values (%)
ECG	41(34.16%)	29(24.16%)
Lipid profile	97(80.83%)	91(75.83%)
HbA1C	103(85.83%)	72(60%)
Foot inspection	46(38.33%)	21(17.5%)
kidney function tests	33(27.5%)	14(11.66%)

values expressed in frequency (percentage)

Table 3:- Age profile of subjects (controls and diabetics) screened.

Age group (years)	Control(n=120)	Diabetic(n=120)
<35	11 (9.16%)	5 (4.16%)
36-45	24 (20%)	15 (12.5%)
46-60	62 (51.66%)	75 (62.5%)
>60	23 (19.16%)	25 (20.83%)
Total	120	120

χ^2 =15.013 df=3, *p*<0.0018

Table 4:- Distribution of subjects (controls and diabetics) according to family history of T2DM.

Family history	Controls	Diabetic	Total
Yes	17 (14.16%)	81 (67.5%)	98
No	103 (85.83%)	39 (32.5%)	142
Total	120	120	240

χ^2=155.594 df=1, *p*<0.0001

Table 5:- Pattern of care and awareness about the disease among the subjects (controls and diabetics)*

Parameter	Control(n=120)	Diabetic(n=120)
Regular follow-up visits	NA	104(86.66%)
Regular use of prescribed drugs	NA	99 (82.5%)
Self-testing of blood glucose at home	NA	32(26.66%)
Compliance with diet recommendations	NA	91(75.83%)
Basic know-how about disease	68(56.66%)	81(67.5%)

(through Print/audio-visual or IT media)		
Knowledge about future complications	52(43.33%)	66(55%)
Positive role of family in disease care	NA	113(94.16%)
Need help in reaching clinic	NA	11(9.16%)

values expressed in frequency (percentage)

Table 6:- Socio-economic and demographic features of subjects

Parameter	**Control(n=120)**	**Diabetic(n=120)**	***P* value**
Economic status (a)LIG **(b)MIG** **(c)HIG**	24(20%) 50(41.66%) 46(38.33%)	32(26.66%) 46(38.33%) 42(35%)	χ^2=3.334, df=2,*p*=0.1888 not significant
Marital status(a)single **(b)Married** **© Widowed**	35(29.16%) 80(66.66%) 5(4.16%)	17(14.16%) 97(80.83%) 6(5%)	χ^2=13.070, df=2,*p*<.0015 statistically significant
Education(a) Junior **(b) Intermediate** **(c) Graduate** **(d) University**	4(3.33%) 31(25.83%) 47(39.16%) 38(31.66%)	18(15%) 29(24.16%) 49(40.83%) 24(20%)	χ^2=19.275, df=3,*p*=.0002 statistically significant
Occupation (a)service **(b) business** **(c) unemployed** **(d) others**	52(43.33%) 26(21.66%) 30(25%) 12(10%)	61(50.83%) 32(26.66%) 6(5%) 21(17.5%)	χ^2=28.892, df=3,*p*<.0001 statistically significant
Smoking (a)No **(b)Current** **(c)Ex-smoker**	70(58.33%) 33(27.5%) 17(14.16%)	98(81.66%) 13(10.83%) 9(7.5%)	χ^2=27.086,df=2,*p*<.0001 statistically significant
Exercise (a) Mild **(b) Moderate** **(c) Vigorous**	87(72.5%) 17(14.16%) 16(13.33%)	101(84.16%) 13(10.83%) 6(5%)	χ^2=9.444,df-2,*p*=0.0089 statistically significant
Drinking (a) No **(b) Sometimes** **(c) Regular**	93(77.5%) 22(18.33%) 5(4.16%)	95(79.16%) 17(14.16%) 8(6.66%)	χ^2=2.979,df=2,*p*=0.2254 not significant

Table 6:- Contd. from page 19

Parameter	**Control(n=120)**	**Diabetic(n=120)**	***P* value**
Cooking medium **(a) Dalda** **(b) refined oil** **(c) mustard oil**	 11(9.16%) 56(46.66%) 53(44.16%)	 8(6.66%) 73(60.83%) 39(32.5%)	χ^2=9.677,df=2,*p*=.0079 statistically significant
Consumption of junk food **(a)rarely** **(b) sometimes** **© frequently**	 5(4.16%) 57(47.5%) 58(48.33%)	 4(3.33%) 72(60%) 44(36.66%)	χ^2=7.527,df=2,*p*=0.0232 statistically significant

**values expressed in frequency (percentage)*

Discussion:-

The present case control study was undertaken to establish the current status of diabetes and its co-morbidities in Kanpur district**.** The study is significant because this population is a fair representative of the phenomenon of internal (rural to urban) migration. Promise of economic uplift and better living conditions lures people from rural to urban areas. The findings of this study try to reveal new vistas in our knowledge of the epidemic of T2DM in study region.

Based on the patient's health profile (table 2), different clinical tests were performed and the results of these tests (table 1) aimed at detecting the presence and extent of any type of co-morbidity. Glycated hemoglobin (HbA1C) is a routinely used marker for long term glycemic control. In accordance with its function as an indicator for the mean blood glucose level, HbA1C predicts the risk for the development of diabetic complications in diabetic patients. Dyslipidemia is defined by alterations in blood lipid levels. The significantly high levels of TG, TC and low levels of HDL-c observed in our subjects probably contribute to insulin resistance (Mooradian, 2009). It is in line with another study done at Naini region of Allahabad which concluded that Hypercholesterolemia, Hypertriglyceridaemia and lipoprotein are the main lipid abnormalities found in diabetes which is risk for coronary artery disease (Smith and Lall, 2008). This is validated by our study as about 24.16% of our patients were hypertensive had abnormal ECG values and were on the brink of developing Coronary Heart disease which is a major co-morbidity attached with diabetes. American Diabetes Association (ADA) has also discussed about the rationale for management of dyslipidemia in Adults with diabetes (Haffner, 1998).

In the present study, it was observed that serum UA level was significantly increasing ($p<0.001$) in T2DM patients as compared to controls. This finding is concurrent with other studies in which hyperuricemia is linked to development of T2DM and its complications especially cardiovascular (Zoppini et al., 2009) and renal complications (Bo et al., 2001; Rosolowsky et al., 2008). Uric acid is a strong endogenous antioxidant that scavenges nitric oxide directly thus decreasing the bioavailability of nitric oxide in vascular smooth muscles and endothelial cells. This promotes endothelial dysfunction enhancing the risk of progression of Coronary vascular disease (Conen et al, 2004; Feig et al., 2008).

Serum creatinine levels in T2DM patients were also significantly higher when compared statistically with controls ($p < 0.05$) indicating the derangement of kidney function. It is believed that one can plot the inverse of creatinine (I/Cr) over time and get a straight line which can thus be used for "monitoring disease progression" (Mitch and Walser, 1986). A study on progression of nephropathy in T2DM pointed out that T2DM is single most common cause of end stage renal disease (ESRD), but decline in kidney function varies among individuals (Rossing et al., 2004).

These findings are further corroborated by a retrospective analytic study, conducted by reviewing the clinical records of the patients with type 2 diabetes who attended the National Diabetes Centre of Sri Lanka from January 2005 to December 2010. It was observed that nephropathy was significantly associated with poor glycemic control, high HbA1c, high fasting blood glucose, high systolic blood pressure (Wijesuriya, 2012). Hence the pathogenesis of diabetic nephropathy is multi-factorial with contribution from various metabolic abnormalities and marked heterogeneity in clinical picture is seen in long-term diabetics.

Obesity is one of the major risk factors for diabetes, yet there has been little research focusing on this risk factor across India (Rao et al., 2011). Despite having lower overweight and obesity rates, India has a higher prevalence of diabetes compared to western countries suggesting that diabetes may occur at a much lower body mass index (BMI) in Indians compared with Europeans (Rao et al., 2011; Mohan and Deepa, 2006). Significantly higher values ($p<0.001$) were observed in our study in diabetics as compared to controls. The increasing incidences of obesity may result in more than a million extra cases of type 2 DM, cardiovascular disease and cancer.

Our study has shown that the population most affected by diabetes is of the age group 46-60 (see table 3). This is corroborated by another study in district Sonepat of Haryana state (Madaan et al., 2014). This is a disturbing finding as pervasion and persistence of diabetes in the most economically productive age group means staggering economic growth of society.

About 67% of diabetic subjects had either one or both parents affected by diabetes (table 4). Upon analysis, a highly significant association ($p<0.0001$) between family history and T2DM was observed. Similar results were reported by other studies (Rao et al., 2010; Shah et al.,1999; Ramachandran et al., 2008; Ravikumar et al., 2011; Patil and Gothankar, 2013). Family history in T2DM is thus a major risk factor in transferring the disease to next generation. It can however be exploited as a preventable tool to avoid diabetes development in early age.

Table 6 shows the similarities and contrasts in various socio-demographic features among controls and diabetics. A study has shown that being married may confer health advantage against type 2 DM as against bachelors, divorcees

and widowers (Cornelis et al 2014). Similar significant trend (p<0.0015) is seen in our study proving that it is a key support mechanism for the subjects.

The role of junk food in diabetes is highlighted by a study on Musmusculusalbinus mice, which were exclusively fed junk food for thirty days. Their body weight and blood sugar levels clearly indicated that fast food enhanced the risk of obesity and diabetes (Wast et al., 2012). Our study points out that most of the diabetics (about 60%) indulge in fast food sometimes while 47.5% of healthy controls do so and the difference was significant (p<0.0232). An interesting contrast is seen when controls are shown to be frequent consumers (48.3%) as against diabetics (36.6%). This can be attributed to the fact that once the disease is diagnosed, patients tend to make healthier food choices. Similarly, majority of the subjects being non-smokers and non-drinkers points towards adoption of a healthier lifestyle especially by diabetics in view of their diseased state.

Edible oils constitute an important part of diet of a person. A study on the effect of edible oils on biochemical parameters of subjects in kharagpur, west Bengal shows that sesame oil followed by mustard oil proved to be most beneficial against diabetes (Dineshkumar et al., 2009). Our findings show that use of refined oil is more prevalent than mustard oil and the difference in consumption pattern is significant (p<0.0079). It is perhaps advisable to revert to our traditional dietary ways to reduce the incidence of diabetes.

Physical activity or exercise has impact on various components of diabetes. Ever increasing urbanization and socio-economic prosperity has led to decline in the physical activity levels of people. Statistically significant values (p<0.0089) from our study indicates the same and is corroborated by many other findings (Bhatti et al., 2007; Williams et al., 1994; Lip et al., 1996; Hughes et al., 1990) which have shown that South Asians and Asian Indians are lesser physically active than other ethnic groups. It is interesting to note that in our study most of the controls as well as patients seem to follow a mild exercise routine. This resistance towards strenuous physical activity tips the balance in favor of strong insulin resistance in diabetics and may pave the way towards impaired glucose tolerance in controls later on. This agrees with a south Indian study which has shown that diabetes prevalence is almost three times higher in individuals with light physical activity compared to those having heavy physical activity (Mohan et al., 2003).

Environmental barriers responsible for limited physical activity include unsafe walking areas, transportation problems, medical conditions and also the attitudes and knowledge of subjects (Dutton et al., 2005).

The care and awareness profile of our subjects (table 5) indicates need for an over-all improvement in the area. However, patients are unaware of the multi-factorial nature of the disease. This highlights the fact that apart from clinical treatment, counseling and education of the patients is important. A similar study from rural Tamaka, Kolar district of Karnataka revealed that 75% of the patients were unfamiliar with the long term diabetic complications and diabetic care (Muninarayana et al., 2010). CURES-9 study in Chennai has shown how increasing awareness and empowerment of community can possibly help in the prevention of diabetes and other non-communicable disorders (Mohan et al., 2005). To reduce the disease burden appropriate government interventions and combined efforts of the society should go hand in hand (2).Government policies may help in creating guidelines on diabetes management, funding community programmes for public awareness about the diabetes risk reduction, availability of medicines and diagnostic services to all sections of community (Verma et al., 2012). A significant landmark of our study is the positive role played by family of more than 90% of the diabetics.

It is directly in line with the results of DAWN2 (Diabetes Attitudes, wishes and Needs) study which involved participants from 17 countries of varying socio-cultural environment. The study revealed that family members of Indian diabetics had the least likelihood of feeling depressed and perceiving significant burden in helping the diseased person (Kovacs et al., 2013).

Conclusion:-

Persistent hyperglycemia, marked dyslipidemia along with the surfacing of co-morbidities like renal impairment and Coronary heart disease are the clinical highlights of our study showing that treatment outcome is far from ideal. These when viewed together with the trends of the social and demographic factors and awareness levels clearly indicate the staggering metabolic profile of the study participants. However the results need to be validated by further studies involving much larger cohort size. Patients require aggressive screening and, multi-factorial approach towards drugs and other supplements is needed for improved glycemic control and tackling other complications

related to T2DM. Patient education and empowerment are key steps in assuring good glycemic control.Priority must be given for creating awareness among the public for motivating them to adhere to the preventive strategies.

Abbreviations:-
BMI- Body Mass Index; TC- Total Cholesterol; TG-Triglycerides; HDL-c-High Density Lipoprotein cholesterol; LDL-c-Low Density Lipoprotein cholesterol; VLDL-c- Very Low Density Lipoprotein cholesterol; BP- Blood Pressure; LIG- Lower Income Group; MIG- Medium Income Group; HIG- High Income Group; ECG- Electrocardiography; HbA1c- Glycated Hemoglobin.

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