OXIDATIVE AND INFLAMMATORY STATUS IN TYPE-2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT CARDIAC COMPLICATIONS

DISSERTATION SUBMITTED FOR

M.D. DEGREE

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THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE

APRIL – 2015

CERTIFICATE

This is to certify that the dissertation titled **"OXIDATIVE AND INFLAMMATORY STATUS IN TYPE-2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT CARDIAC COMPLICATIONS"** is an original work done by **Dr.K.INDHU,** PG student, PSG Institute of Medical sciences and Research, Coimbatore, under my supervision and guidance.

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DECLARATION

I solemnly declare that this dissertation work **"OXIDATIVE AND INFLAMMATORY STATUS IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT CARDIAC COMPLICATIONS"** was done and written by me in the Department of Biochemistry, PSG Institute of Medical sciences & Research, Coimbatore, under the guidance of **Prof. Dr.G.Jeyachandran,** M.D, Professor and Head of the Department, Biochemistry, PSG Institute of Medical sciences & Research, Coimbatore.

This dissertation is submitted to the Tamil Nadu Dr. M. G. R Medical University, Chennai in partial fulfillment of the university regulations for the degree of M.D Biochemistry – Branch XIII examinations to be held in April 2015.

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I wish them all good health and long life!

Dr.K.Indhu

ABBREVIATIONS

AGE	-	Advanced glycation end product
CVD	-	Cardio Vascular Disease
DM	-	Diabetes mellitus
DKA	-	Diabetic Ketoacidosis
DAG	-	Diacyl glycerol
FPG	-	Fasting Plasma Glucose
GDM	-	Gestational diabetes mellitus
GLUT	-	Glucose transporter
GSH	-	Glutathione
HbA1c	-	Glycated hemoglobin
hs-CRP	-	High-sensitivity C-reactive protein
H_2O_2	-	Hydrogenperoxide
OH·	-	Hydroxyl radical
IFG	-	Impaired fasting glucose
IGT	-	Impaired glucose tolerance
LDL	-	Low Density Lipoprotein
MODY	-	Maturity onset diabetes of the young
MHC	-	Major histocompatability complex
NOS	-	Nitric oxide synthase
PAI	-	Plasminogen activator inhibitor
ROO·	-	Peroxyl radical
PKC	-	Protein kinase C
ROS	-	Reactive Oxygen Species
SOD	-	Superoxide dismutase
O_2^{-}·	-	Superoxide anion
–SH	-	Sulfhydryl group
TNF	-	Tumor necrosis factor
VCAM	-	Vascular cell adhesion molecule
VLDL	-	Very-low density lipoproteins

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September 4, 2013

To
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The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on 23rd August, 2013 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 2.00 pm and 3.30 pm, and discussed your study proposal entitled:

"Oxidative and inflammatory status in type 2 diabetes mellitus patients with and without cardiac complications"

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Informed Consent Forms (Ver 1.1)
4. Data Collection Tool
5. Budget
6. CV

After due consideration, the Committee has decided to approve the above study.

The members who attended the meeting, at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr D Vijaya	Ph D	Member – Basic Scientist	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member – Social Scientist	Male	Yes	Yes

The approval is valid for one year.

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We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R and also, after completion of the project, please submit completion report to IHEC.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Yours truly,

4/9/17

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

SECRETARY
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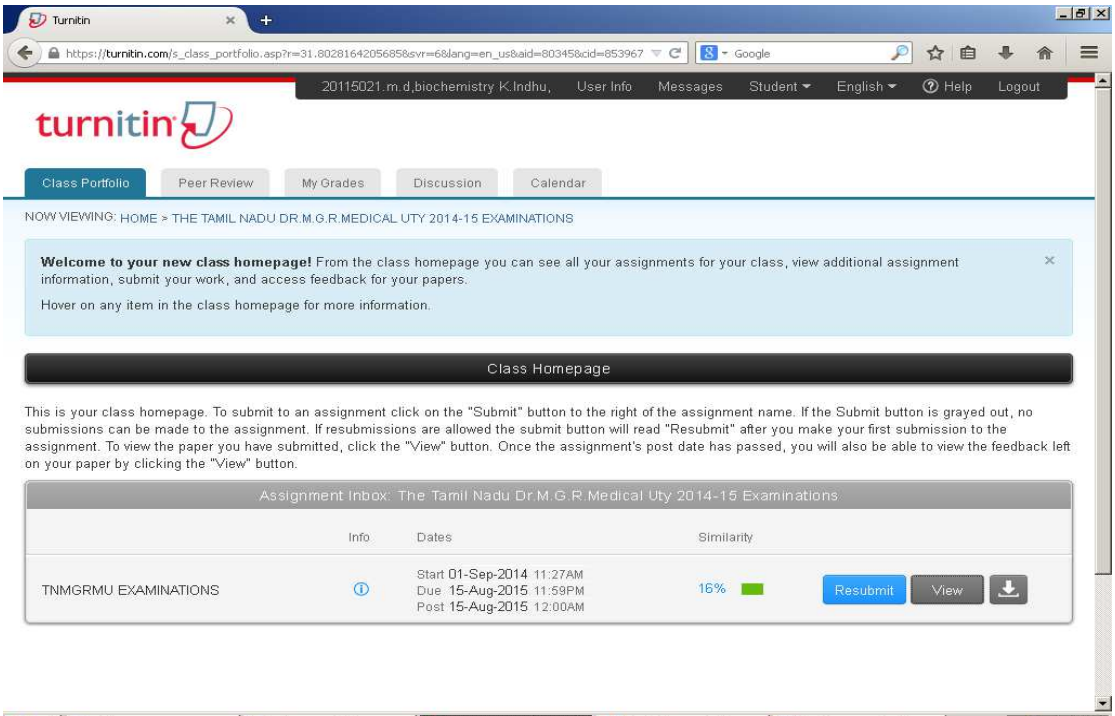
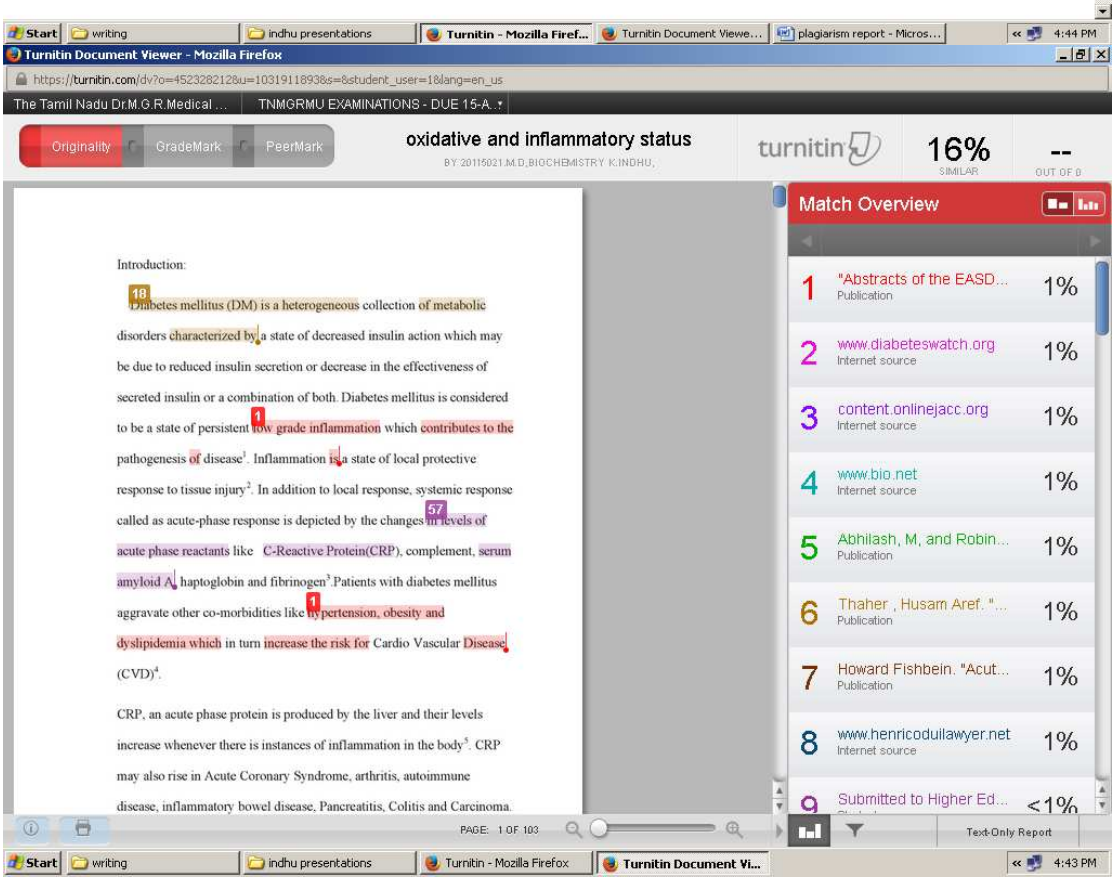
PLAGIARISM REPORT:





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OXIDATIVE AND INFLAMMATORY STATUS IN TYPE-2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT CARDIAC COMPLICATIONS

ABSTRACT

Background and Objective of the study:

Diabetes mellitus, a heterogeneous collection of metabolic disorders may be due to reduced insulin secretion or decrease in the effectiveness of secreted insulin or a combination of both. Increased oxidative stress and inflammation contributes to the development and progression of diabetes and its complications. Diabetes aggravates other co-morbidities such as obesity, hypertension and dyslipidemia which also increase the risk for Cardio Vascular Disease. High-sensitivity C-reactive protein (hs-CRP) an acute phase protein is considered to be predictor of future coronary events. Thiols constitute the major portion of the total body antioxidants and they play a significant role in defense against reactive oxygen species.

Objective of this study was to estimate the levels of hsCRP and protein thiols in type 2 diabetic patients with and without cardiac complications .In addition this study also tries to establish a correlation between glycated haemoglobin, hsCRP and protein thiols in both the study groups.

Material and Methods:

This is a cross sectional study. Type-2 diabetic patients belonging to the age group of 30-75 years were selected from Diabetology OPD and Cardiology ward. HbA1c and plasma hsCRP were estimated in 60 type-2 diabetic patients without complications and 60 type-2 diabetic patients with cardiac complications. Type-2 diabetic patients with other complications were excluded. Plasma total protein thiols was estimated spectrophotometrically by using Dinitrobenzene (DTNB)-Ellman's method, plasma hsCRP was measured by particle enhanced turbidimetric assay and HbA_1c by turbidimetric inhibition immunoassay.

Results:

There was no statistically significant difference between the two study groups with regard to age and gender. The mean hsCRP activity in type-2 diabetic patients

without complications was 0.2885 ±0.26758 and with cardiac complications was 3.1970±5.83335 which was statistically significant with the p value of 0.000187. The p value of HbA1c levels between the two groups was found to be 0.047 which was also statistically significant. There was no statistical significance (p value, 0.530) between the two groups with regard to protein thiols. Further, the Pearson correlation analysis showed a significant positive correlation between the blood levels of HbA1c with the plasma total protein thiols (p value < 0.001) and hsCRP (p value 0.018). In addition there is a very significant positive correlation between the plasma levels of hsCRP and the plasma total protein thiols with a p value of < 0.001.

Interpretation and Conclusion:

Increased HbA1c and plasma hsCRP in type 2 diabetic patients with cardiac complications can be attributed to the fact that oxidative stress and inflammation due to persistent hyperglycemia play a major role in the pathogenesis of diabetic complications. The increase in thiol levels along with hsCRP is due to the increased synthesis to compensate for the loss incurred during neutralization of the oxidants in Type 2 diabetic patients.

Key words: Diabetes mellitus, Cardiovascular disease, Atherosclerosis, Oxidative stress, Inflammation, High sensitivity C reactive protein, Protein thiols

1. Introduction:

Diabetes mellitus (DM) is a heterogeneous collection of metabolic disorder characterized by a state of decreased insulin action which may be due to reduced insulin secretion or decrease in the effectiveness of secreted insulin or a combination of both. Diabetes mellitus is considered to be a state of persistent low grade inflammation which contributes to the pathogenesis of disease[1]. Inflammation is a state of local protective response to tissue injury[2]. In addition to local response, systemic response called as acute-phase response is depicted by the changes in levels of acute phase reactants like C-Reactive Protein(CRP), complement, serum amyloid A, haptoglobin and fibrinogen[3].Patients with diabetes mellitus aggravate other co-morbidities like hypertension, obesity and dyslipidemia which in turn increase the risk for Cardio Vascular Disease (CVD)[4].

CRP, an acute phase protein is produced by the liver and their levels increase whenever there is instances of inflammation in the body[5]. CRP may also rise in acute Coronary Syndrome, arthritis, autoimmune disease, inflammatory bowel disease, pancreatitis, colitis and carcinoma. CRP testing cannot be used to diagnose specific diseases but serves more as a general indicator of inflammation or infection[6]. Numerous epidemiologic studies done in United States and Europe have concluded

high-sensitivity C-reactive protein (hs-CRP) to be a predictor of future coronary events among apparently healthy individuals[7].

Increased oxidative stress plays a major role in the progression of diabetes and development of complications[8].Oxidative stress increases when the rate of free radical production is increased and/or the antioxidant mechanisms are impaired[9]. Free radicals are unstable species which are produced continuously during aerobic metabolism. These free radicals cause oxidative damage to carbohydrates, proteins, lipids and DNA that are normally neutralized by protective antioxidants. The imbalance between increased free radical production and protective antioxidants to neutralize it, leading to oxidative damage is known as oxidative stress. Studies have demonstrated reduced concentration of vitamin A, C, E and the antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase in type 2 diabetics[10, 11]. Incomplete scavenging of reactive free radicals causes oxidation of cellular proteins, lipids, nucleic acids and glycoconjugates which in turn leads to fragmentation and cross-linking causing extensive pathological consequences leading to cell death. Oxidation of glucose and glycosylated proteins in type 2 diabetic patients is thought to be the reason for increased production of damaging free radicals[12].

Thiols are the organic compounds that contain a sulfhydryl group (-SH). Thiols form the major portion of the total body antioxidants among all other antioxidants and they play an important role in defense against reactive oxygen species[13].The reduced thiol (–SH) groups can exist both intracellularly and extracellularly in two forms. They can be either in free form as reduced glutathione or can exist as protein bound thiols playing a major role in conserving the antioxidant status of the body[14]. Among the protein bound thiols, albumin makes the major portion of it, which binds to sulfhydryl group at its cys-34 portion[15]. In oxidative stress, protein oxidation products are formed early, have greater stability and longer lifespan than Reactive Oxygen Species (ROS) and lipid peroxidation products. Hence, they are being increasingly used to demonstrate oxidative stress in place of lipid peroxidation[16].

This study is done to estimate the levels of hsCRP and protein thiols in type 2 diabetic patients with and without cardiac complications .In addition we also have tried to establish a correlation between glycated haemoglobin, hsCRP and protein thiols in both the study groups.

2. Aims and Objectives

Aim:

To evaluate the oxidative and inflammatory status in type 2 diabetic patients with cardiac complications and compare the same with type 2 diabetic patients without cardiac complications

Objectives:

1. To estimate the levels of oxidative marker (Protein thiols) and inflammatory marker (hsCRP) in type 2 diabetes mellitus patients with and without cardiac complications.

2. To find out the correlation between oxidative, inflammatory markers and glycated hemoglobin (HbA1c) in the study groups.

3. Review of literature:

Introduction:

Diabetes mellitus is a group of metabolic diseases presenting with signs and symptoms of hyperglycemia which may be due to defects in insulin secretion, insulin action, or both.

Epidemiology of diabetes mellitus:

Global prevalence of diabetes mellitus in adult population (20-79 years old) is estimated to be around 8.3% with 382 million people suffering from diabetes .North America and the Caribbean region has got the highest prevalence of disease (11%) followed by the Middle East and North Africa (9.2%)[17]. Type 2 DM accounts for approximately half of adolescent diabetes in the United States, and one-third of these cases were undiagnosed[18]. It was estimated that nearly 1 million Indians die due to diabetes every year with the average age of onset being 42.5 years and it is expected that by 2030 incidence will increase possibly due to increased prevalence of obesity and lack of physical activities[19, 20]. Prevalence of diabetes mellitus is higher in men less than 60 years of age when compared to women at older ages[21]. Majority of people with diabetes are in 45 to 64 years of age in developing countries, whereas in developed countries most of them are greater than 64 years of age[22]. The prevalence

of Type 2 diabetes is high and it is 4-6 times higher in urban than in rural areas of india[23].

Classification:

Diabetes mellitus rather than being classified on the basis of age at which diabetes has been diagnosed and therapy given to them is now classified according to the pathogenesis of disease that leads to increase in blood glucose levels.

Etiological classification of diabetes mellitus[24]:

- Type I diabetes mellitus is due to β-cell destruction, leading to complete or near total insulin deficiency
- Type II diabetes mellitus is due to variable degrees of resistance to insulin action and decreased insulin secretion
- Gestational diabetes mellitus (GDM) – intolerance to glucose developed during the course of pregnancy
- Other types

A. Impairment in beta cell function due to genetic defects

1. Hepatocyte Nuclear Factor -1a, Maturity onset diabetes of the young (MODY3)
2. Glucokinase (MODY2)

3. Hepatocyte Nuclear Factor-4a (MODY1)

4. Insulin promoter factor-1 (MODY4)

5. Hepatocyte Nuclear Factor -1b (MODY5)

6. NeuroD1 (MODY6)

7. DNA of mitochondrial origin

8. Other genetic defects

B. Genetic defects leading to defective insulin action

1. Type A insulin resistance

2. Leprechaunism

3. Rabson-Mendenhall syndrome

4. Lipoatrophic diabetes

5. Other genetic defects

C. Diseases of the exocrine pancreas

1. Pancreatitis

2. Trauma to pancreas

3. Malignancy

4. Cysticfibrosis

5. Hemochromatosis

6. Fibrocalculous pancreatopathy

7. Following Pancreatectomy

D. Endocrinopathies

1. Acromegaly
2. Cushing's syndrome
3. Glucagonoma
4. Pheochromocytoma
5. Hyperthyroidism
6. Somatostatinoma
7. Aldosteronoma
8. Other disorders

E. Drug or chemical induced

1. Vacor
2. Pentamidine
3. Nicotinic acid
4. Corticosteroids
5. Thyroid hormone
6. Diazoxide
7. β-adrenergic agonists
8. Thiazides
9. Dilantin
10. γ-Interferon
11. Other drugs

F. Infections

1. Congenital rubella
2. Cytomegalovirus
3. Other infections

G. Immune-mediated forms of diabetes

1. Stiff-man syndrome
2. Anti-insulin receptor antibodies
3. Other forms

H. Other genetic syndromes sometimes associated with diabetes

1. Down syndrome
2. Klinefelter syndrome
3. Turner syndrome
4. Wolfram syndrome
5. Friedrich ataxia
6. Huntington chorea
7. Laurence-Moon-Biedl syndrome
8. Myotonic dystrophy
9. Porphyria
10. Prader-Willi syndrome

Criteria for diagnosing diabetes mellitus[25]:

- Glycated hemoglobin ≥ 6.5%. or
- Fasting Plasma Glucose (FPG) ≥ 126 mg/dL or
- 2-hour plasma post prandial glucose ≥ 200mg/dL during an Oral Glucose Tolerance Test or
- Random plasma Glucose ≥ 200 mg/dL with signs and symptoms of hyperglycemia

Categories with increased risk for diabetes[25]:

- Impaired fasting glucose[IFG]:FPG 100 mg/dL to 125 mg/dL
- Impaired glucose tolerance [IGT]:2-hour plasma glucose in the 75g oral glucose tolerance test 140 mg/dL to 199 mg/dL
- Glycated hemoglobin - 5.7-6.4%

Type 1 diabetes mellitus:

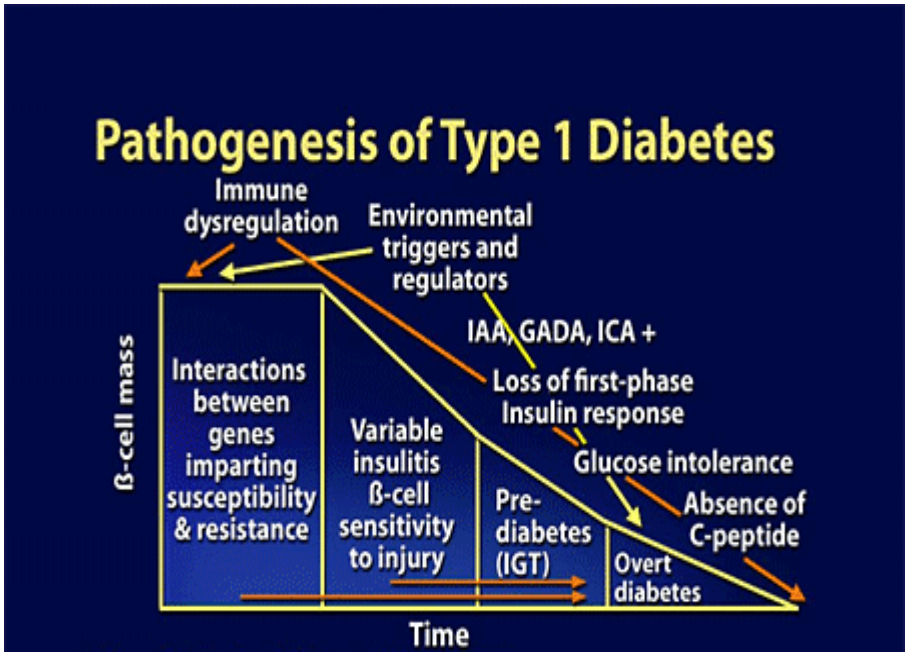
Type 1 diabetes mellitus primarily due to beta cell destruction can be subdivided into

- Immune etiology, Type 1A
- Unknown etiology, Type 1B

Type 1 diabetes mellitus is due to autoimmune destruction of pancreatic beta cells leading to insulin deficiency. The rate of beta cell destruction varies among individuals. The major vulnerable gene is the Human Leukocyte Antigen gene which codes for major histocompatability complex (MHC) class II on chromosome 6 for type 1 diabetes mellitus. Class II MHC initiates immune response by presenting antigens to helper T cells which depends on the composition of amino acids on the antigen binding site[26]. In addition to MHC II, multiple gene polymorphisms have been reported to increase the risk of type 1A diabetes. These include interferon-induced helicase , preproinsulin, Protein tyrosine phosphatase nonreceptor type 22 gene PTPN22, Cytotoxic T Lymphocyte Antigen -4, Interleukin 2 receptor (CD25), a lectin-like gene (KIA0035), epidermal growth factor receptor family ERBB3e, and undefined gene at 12q.Variations in the number of nucleotide repeat elements 5` of the insulin gene, PTPN22 gene encoding

a lymphoid specific phosphatase that influences T cell receptor signaling are all associated with the development of type 1A diabetes[27].Greater than 90% of the patients are positive for auto antibodies to islet cells. They include autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA) and islet cells (ICA) [28].

Fig 3.1: Pathogenesis of type 1 diabetes mellitus



Source: Atkinson MA, Eisenbarth GS. Type 1 diabetes: New perspectives on disease pathogenesis and treatment. Lancet 2001; 358: 221–229.

Type 2 diabetes mellitus:

Type 2 DM is the most prevalent form of diabetes. It affects greater than 90% of the population suffering from diabetes globally. There is a rapid increase in the number of diabetic patients and this fiery growth is noted in both rural and urban areas. It is characterized by excessive hepatic glucose production, variable degree of resistance to insulin action, decreased insulin secretion, and abnormalities in fat metabolism.

Risk factors for type 2 diabetes mellitus[25]:

Sedentary life, lack of physical activity, diet, lifestyle changes and related epidemiological conversion has been established as risk factors for type 2 DM. Other major risk factors are listed below.

- Family history of diabetes mellitus
- Overweight with Body Mass Index, BMI ≥ 25 kg/m^2
- Decreased physical activity
- Ethnicity
- Previously identified to have IFG/IGT
- Blood pressure $\geq$ 140/90 mm of Hg
- Triglycerides $\geq$ 250 mg/dL
- High Density Lipoprotein (HDL) $\leq$ 35 mg/dL
- Previous History of Gestational diabetes mellitus

- Polycystic ovary syndrome
- Acanthosis nigricans

Pathogenesis of type 2 Diabetes Mellitus:

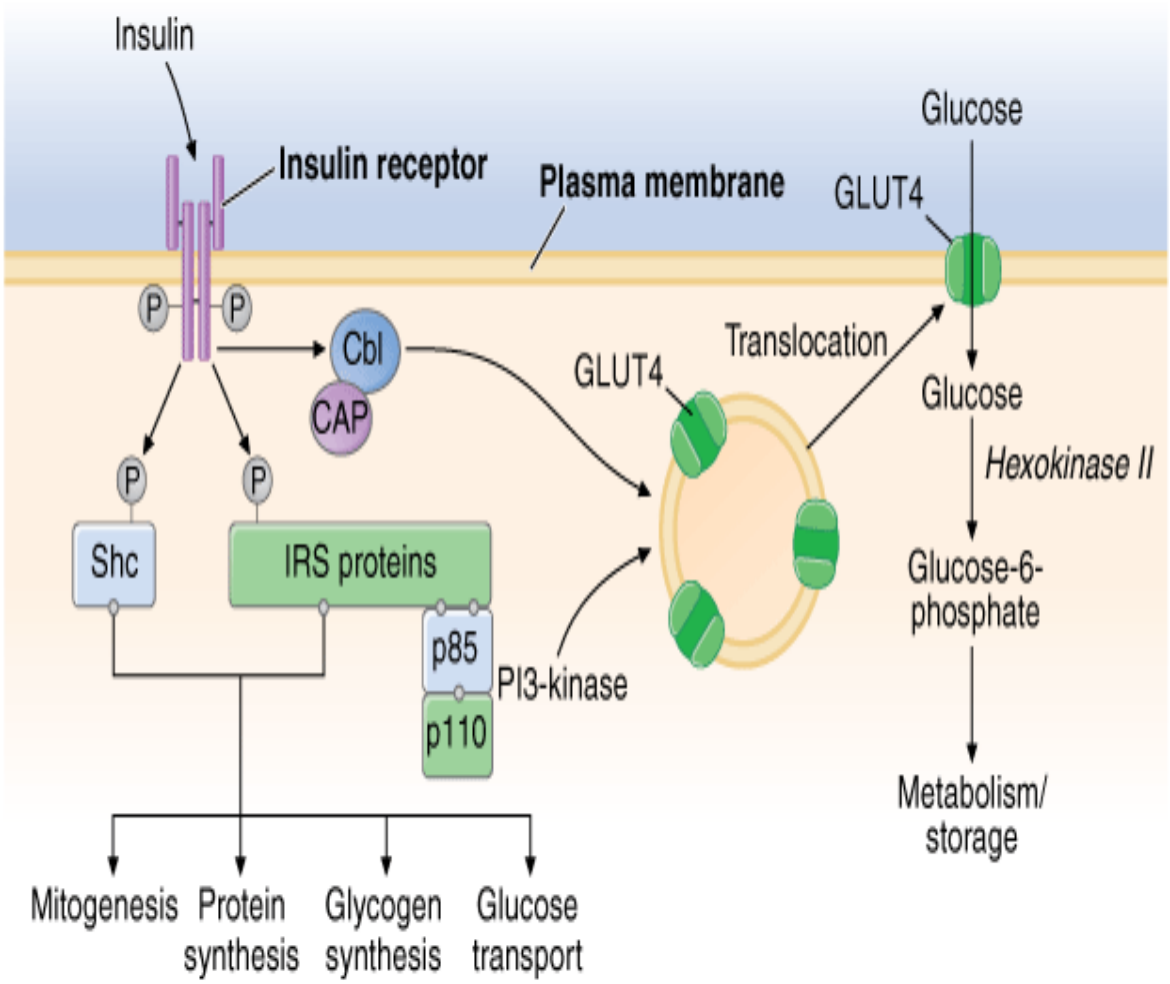
Type 2 DM is a progressive multifactorial disease, with insulin resistance and decreased activity of beta cells playing major role in the pathogenesis of disease.

Insulin resistance refers to impaired response of the body to either exogenously administered insulin or to insulin secreted by the pancreatic beta cells endogenously. This is manifested as decrease in insulin stimulated glucose transport, metabolism in skeletal muscle and adipocytes and by defective suppression of hepatic production of glucose, all these leading to hyperglycemia. Genetic susceptibility and obesity predisposes to insulin resistance. Mutation in the insulin receptor may also interfere with insulin signal transduction pathway.

Decreased response of the target cells to insulin leads to hyperinsulinemia which in turn reduces insulin receptor level and tyrosine kinase activity. Defect in phosphatidyl inositol 3- kinase signaling reduces Glucose transporter4 (GLUT4) translocation to plasma membrane. There is reduced insulin stimulated mitochondrial Adenosine

Tri Phosphate (ATP) production due to lipid accumulation in skeletal muscle which leads to impairment in mitochondrial oxidative phosphorylation. Reactive oxygen species are generated due to impaired fatty acid oxidation and lipid accumulation within skeletal myocytes. Increase in free fatty acids due to increased adipocyte mass, impairs glucose utilization in skeletal muscle, promotes hepatic gluconeogenesis and impairs beta cell function. Cytokines secreted by adipose tissue contributes to the rise in IL-6 and CRP in type 2 DM[29].

Fig 3.2: Insulin signal transduction pathway in skeletal muscle

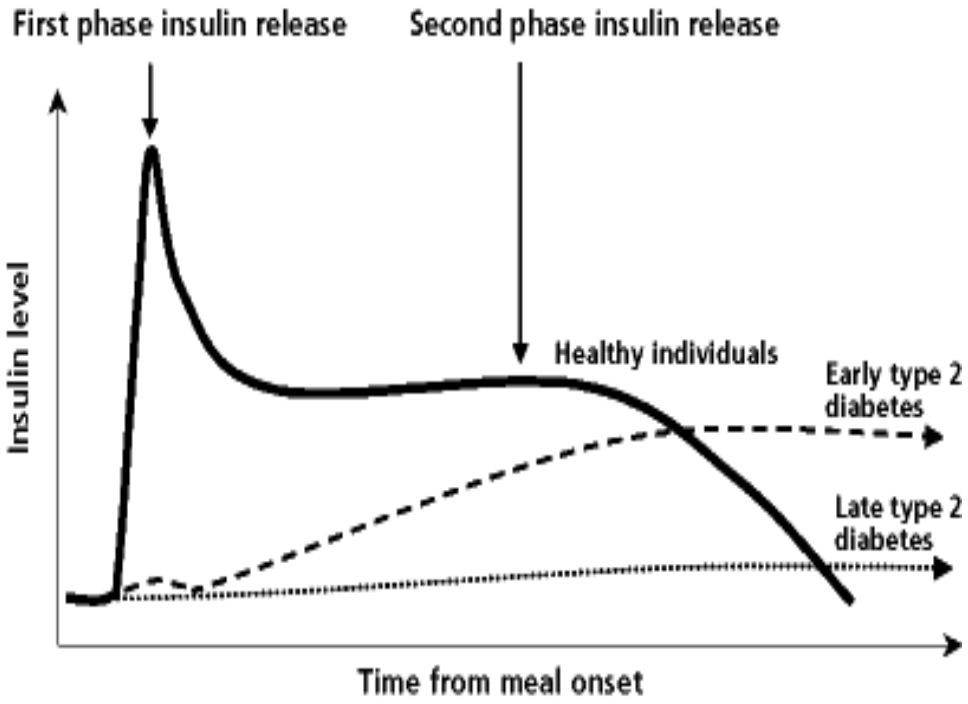


Source: Dan Longo,Anthony Fauci,Dennis Kasper,Stephen Hauser. Harrison's Principles of Internal Medicine.18th edition .chapter 344, Diabetes mellitus: fig 344-5, 2972

Impaired insulin secretion:

Variations in blood glucose concentration activate the beta-cells of the pancreatic islets to secrete insulin. In response to a rapid increase in blood glucose concentration, insulin is released from the beta-cells of pancreas in a biphasic pattern (fig 3.3). The first phase in insulin secretion lasts only for few minutes followed by a slower and steadily evolving second phase, which lasts till the glucose level remains elevated. Conversely, a slow increase in plasma glucose level induces a progressively larger secretion without the first phase of insulin secretion. Type 2 diabetic patients has a substantially lower first phase of insulin secretion than the healthy subjects, and often it may be absent. The second phase of insulin secretion is also lower than healthy subjects[30].

Fig 3.3: Phases of insulin secretion

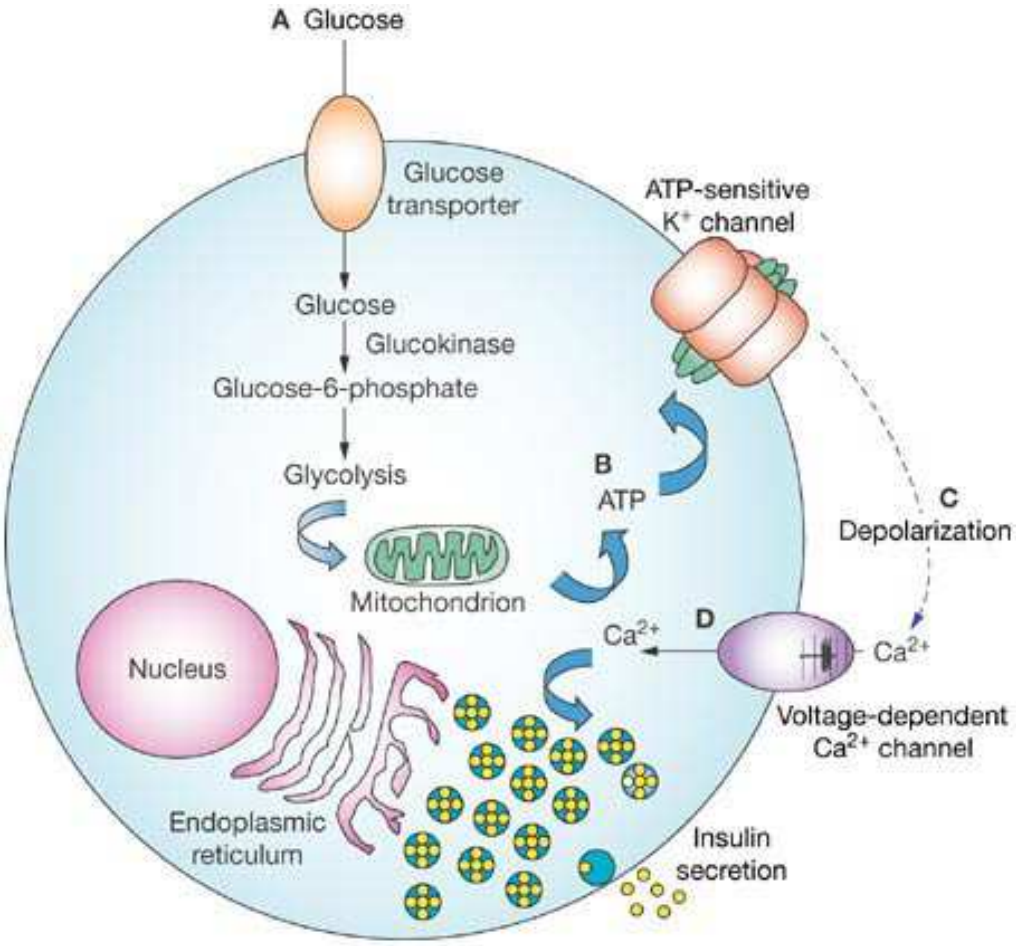


Modified from Lupi R, Del Prato S. Beta-cell apoptosis in Type II diabetes: quantitative and functional consequences. Diabetes Metab. 2008 Feb; 34 Suppl 2:S56-S64

By diffusion, glucose enters the pancreatic beta cells through GLUT2 transporters and stimulates them to secrete insulin. The pancreatic beta-cell metabolises glucose to produce ATP, and the increase in ATP/ADP ratio favours the closure of ATP-sensitive K^+ channels in the cell surface which causes depolarization of the cell-membrane. As a result of depolarization, voltage-dependent Ca^{2+} channels are opened

facilitating the entry of extracellular Ca^{2+}into the beta-cells. The rise in cytosolic Ca^{2+} inside the beta cells triggers the release of insulin[29].

Fig 3.4: Glucose mediated insulin secretion in beta cells of pancreas



Source: Diva D De León and Charles A Stanley .Mechanisms of Disease: advances in diagnosis and treatment of hyperinsulinism in neonates.Nature Clinical Practice Endocrinology & Metabolism 2007; 3:57-68

Elevated free fatty acids and chronic hyperglycemia also contributes to the worsening of islet function. Resistance to insulin action in adipose tissue leads to lipolysis and increased free fatty acids in turn leads to increased synthesis of very low density lipoprotein and triglyceride in hepatocytes. These changes are the reason for dyslipidemia in type 2 diabetes mellitus. The three main mechanisms leading to augmented beta-cell apoptosis are chronic hyperglycemia, lipotoxicity, and islets amyloid polypeptide (IAPP) deposition. Insulin secretion in type 2 diabetes is also affected by genetic variants through their effects on conversion of proinsulin, release of insulin on glucose stimulation, incretin secretion or sensitivity to incretin action, proliferation of beta cells and apoptosis[26].

Complications of diabetes mellitus[26]:

Acute complications:

Ketoacidosis

Hyperglycemic hyperosmolar nonketotic syndrome

Hypoglycemia

Chronic complications include

- **Microvascular**

  Eye disease

    Retinopathy (nonproliferative/proliferative)

    Macular edema

  Neuropathy

    Sensory and motor (mono- and polyneuropathy)

    Autonomic

    Nephropathy

- **Macrovascular**

  Coronary artery disease

  Peripheral arterial disease

  Cerebrovascular disease

- **Others**

  Gastrointestinal dysfunction such as gastroparesis, diarrhea

  Genitourinary abnormalities such as uropathy/sexual dysfunction

Dermatological conditions

Infections

Cataract

Glaucoma

Periodontal disease

Acute complications of DM:

Diabetic Ketoacidosis (DKA):

DKA is characterized by absolute lack of insulin with blood glucose levels usually >200 mg/dL, increased free fatty acid levels, increased production of ketone body, raised ketone body levels in blood and acidosis (pH ≤ 7.3).Patients with DKA presents with acute abdominal pain, kussmaul`s breathing, dehydration with signs and symptoms of hyperglycemia. In patients suffering from diabetes mellitus precipitating factors for DKA include the following

- Bacterial and viral Infections
- Acute illness
- Lack of awareness on diabetes education

- Non-compliance, reduced self-care
- Inadequate monitoring of glucose
- Psychiatric problems

Morbidity and mortality associated with DKA depends on the severity of electrolyte and acid-base disturbances leading to coma and death[31].

Hyperosmolar non-ketotic coma:

It is defined by the presence of hyperglycemia due to relative insulin deficiency with blood glucose level usually >1000 mg/dL with elevated serum osmolality >300 mosm/kg, signs of dehydration and stupor. If not corrected it may progress to coma with no evidence of ketosis or acidosis. This is because these patients have sufficient amount of insulin to prevent ketosis and lipolysis. The usual precipitating factors are medications such as corticosteroids, thiazide diuretics, dehydration, acute illness, infections,cerebral vascular disease and old age[32].

Hypoglycemia:

Hypoglycemia commonly occurs in diabetic patients on treatment with insulin and it may also occur in diabetic patients treated with the oral hypoglycemic agents such as sulfonylureas. Hypoglycemia varies from blood glucose level of 60-70 mg/dL with minimal or no symptoms, to

severe hypoglycemia with blood glucose level of less than 40 mg/dL along with neurologic impairment. Oral carbohydrates are sufficient to manage glucose levels of 40-70 mg/dL and no other medical intervention is required whereas glucose levels less than 40 mg/dL requires further medical intervention with intravenous glucose or glucagon.

Precipitating factors include

- Drug dosage errors
- Human insulin use
- Secretion of counter regulatory hormones can be impaired
- Heavy Exercise
- Skipping meals or delayed meals
- Intensity at which glycemic control is acheived
- Absorption of insulin from subcutaneous depots may vary
- Insulin binding to receptors, insulin action, rate of degradation may vary

Renal, adrenal and pituitary insufficiency contributes to the increased frequency of hypoglycemic events in diabetic patients[33].

Chronic complications of DM [34]:

Diabetes mellitus being a chronic disease affects multiple organs and they contribute to majority of morbidity and mortality in these patients.

Chronic complications are of two types

- Vascular
- Nonvascular

The vascular complications are further divided into

- Microvascular which include retinopathy, neuropathy, and nephropathy
- Macrovascular complications which include coronary artery disease, peripheral vascular disease, cerebrovascular disease.

Diabetic retinopathy:

It is the most frequently occurring microvascular complication and its occurrence depends on the duration and severity of diabetes mellitus. Increase in blood glucose levels increases the entry of sugar molecules through the polyol pathway, which converts glucose to sorbitol leading to its accumulation within the cells. Sorbitol accumulation within the cells causes osmotic stress which is believed to be the cause for basement

membranes thickening, microaneurysm formation and pericyte loss. Growth hormone, vascular endothelial growth factor (VEGF) and transforming growth factor β, have also been known to play important roles in the progression of diabetic retinopathy[34].

Diabetic nephropathy:

Diabetic nephropathy is one of the important causes of renal failure. Microalbuminuria is a condition where albumin excretion is between 30-299 mg/day. Microalbuminuria in diabetic patients if not treated may progress to massive proteinuria and then leading to diabetic nephropathy. Similar to diabetic retinopathy, there is a strong association between glycemic control and the risk of developing diabetic nephropathy. The pathological changes in the kidney include increase in the thickness of glomerular basement membrane, formation of microaneurysms and mesangial nodules called as Kimmelsteil-Wilson bodies. Albuminuria occurring in patients with type 2 diabetes mellitus may also be due to other diseases like hypertension, congestive heart failure, prostate disease and infection[34].

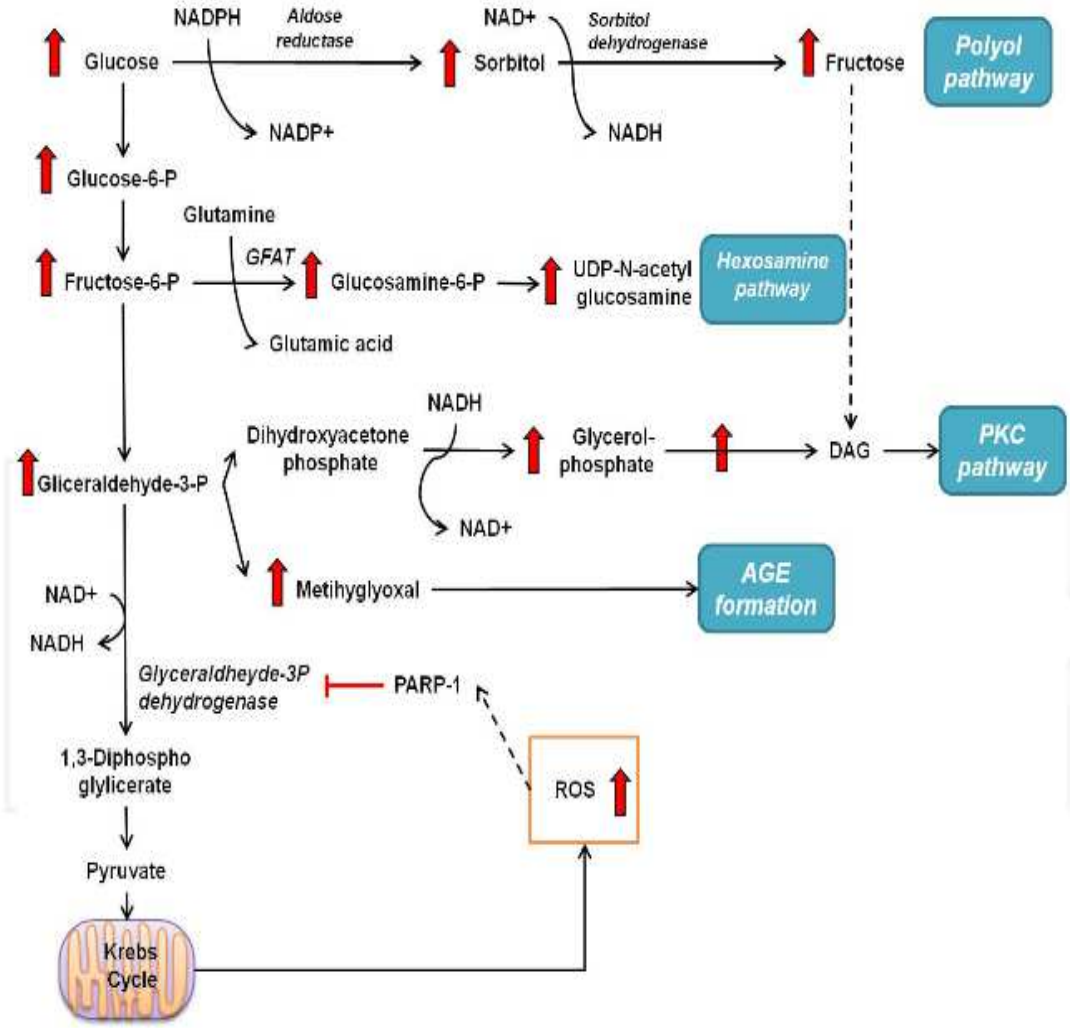
Diabetic neuropathy:

Diabetic neuropathy presents with signs and symptoms of peripheral nerve dysfunction in people with diabetes after other causes have been excluded. Development of diabetic neuropathy also depends on the duration and severity of diabetes mellitus. Neuropathy in diabetics manifests as polyneuropathy , mononeuropathy, and/or autonomic neuropathy. Chronic distal symmetric sensorimotor polyneuropathy is the most frequent form of neuropathy in diabetes[34].

Pathogenesis of microvascular complications:

Microvascular disease occurs predominantly in tissues where uptake of glucose is not dependent on insulin activity such as retina, vascular endothelium and renal cells. These insulin independent tissues are continuously exposed to increased glucose levels in diabetic patients. Glucose-mediated endothelial damage, overproduction of superoxides leading to oxidative stress, production of sorbitol and advanced glycation end-products due to hyperglycaemic state (fig 3.5) contributes to the development of microvascular disease. These metabolic insults alter the rate of blood flow and causes change in endothelial permeability, deposition of extravascular protein and coagulation abnormalities ensuing organ dysfunction.

Fig 3.5: Pathobiology of diabetic complications



Source: Michael Brownlee. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813-820

Macrovascular complications of Diabetes:

The socio economic burden of diabetes mellitus is mainly attributed to its macrovascular complications. The basic pathology behind the occurrence of macrovascular disease is atherosclerosis.

Atherosclerosis, a multifactorial disease is characterized by cholesterol accumulation, infiltration of macrophages, smooth muscle cell proliferation, accumulation of connective tissue components and thrombus formation.

Pathogenesis of atherosclerosis:

Atherosclerotic lesions develop under an intact endothelium but when the integrity of the endothelium is altered. Proteins and lipoprotein particles extravasate through the defective endothelium into the subendothelial space.

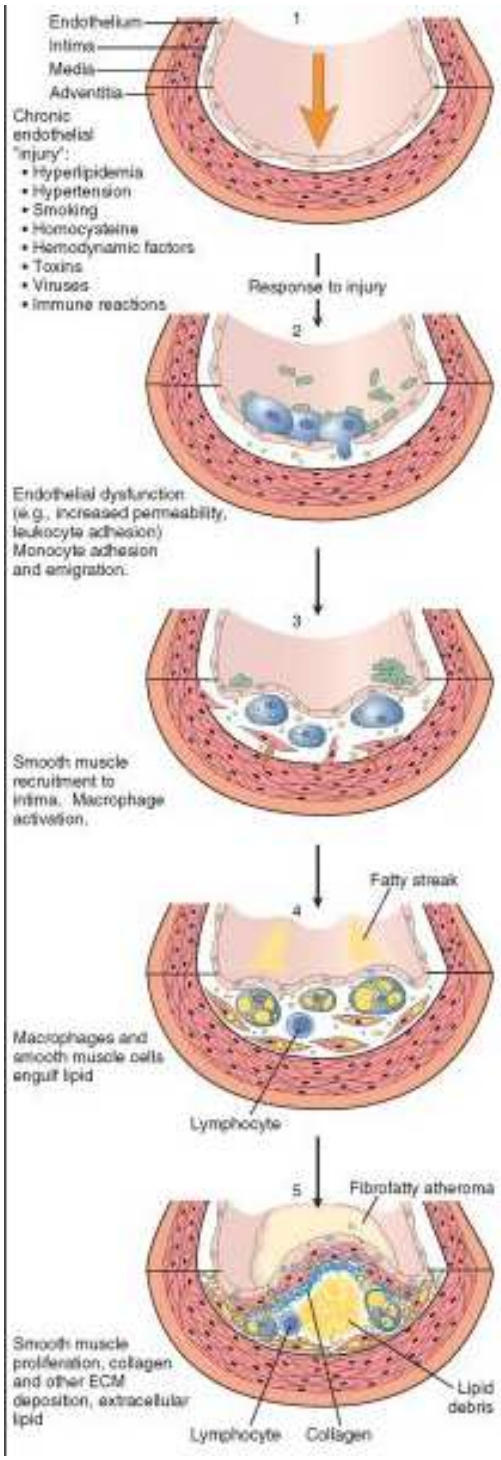
Atherogenic modification of Low Density Lipoprotein (LDL) is mediated by myeloperoxidase, 15-lipoxygenase and nitric oxide synthase (NOS). This modified LDL is pro-inflammatory, chemotaxic and proatherogenic. Nitric oxide produced by inducible NOS in macrophages, is potentially proatherogenic. Atherogenic stimuli such as elevated cholesterol, smoking and proinflammatory stimuli activates the endothelium and upregulates the expression of adhesion molecules

primarily vascular cell adhesion molecule-1(VCAM-1), followed by recruitment of monocytes and T cells. In addition to VCAM-1, other adhesion molecules, such as intercellular adhesion molecule-1, E selectin, and P selectin also have a say in the recruitment of blood cells to the atherosclerotic site.

The first cellular responses to occur in atherogenesis are the focal recruitment of circulating monocytes followed by T lymphocytes to a lesser extent. There is an increase in Monocyte chemotactic protein-1, a powerful chemokine and its receptor on monocytes and macrophages during plaque development and is necessary for trans-endothelial migration of cells. Macrophages, endothelial cells and smooth muscle cells contribute to the over expression of this chemokine in the process of atherosclerosis. Foam cells are the hallmark of atherosclerotic lesions. Lipid-laden macrophages containing cholesteryl esters in abundance are called foam cells. Apoptosis and necrosis of macrophages contributes to the formation of a lipid-rich necrotic core which is soft and destabilizing within the atherosclerotic plaque. Smooth muscle cells and the collagen enriched matrix retrieves stability to plaques thereby protecting them from plaque rupture and thrombosis[35].

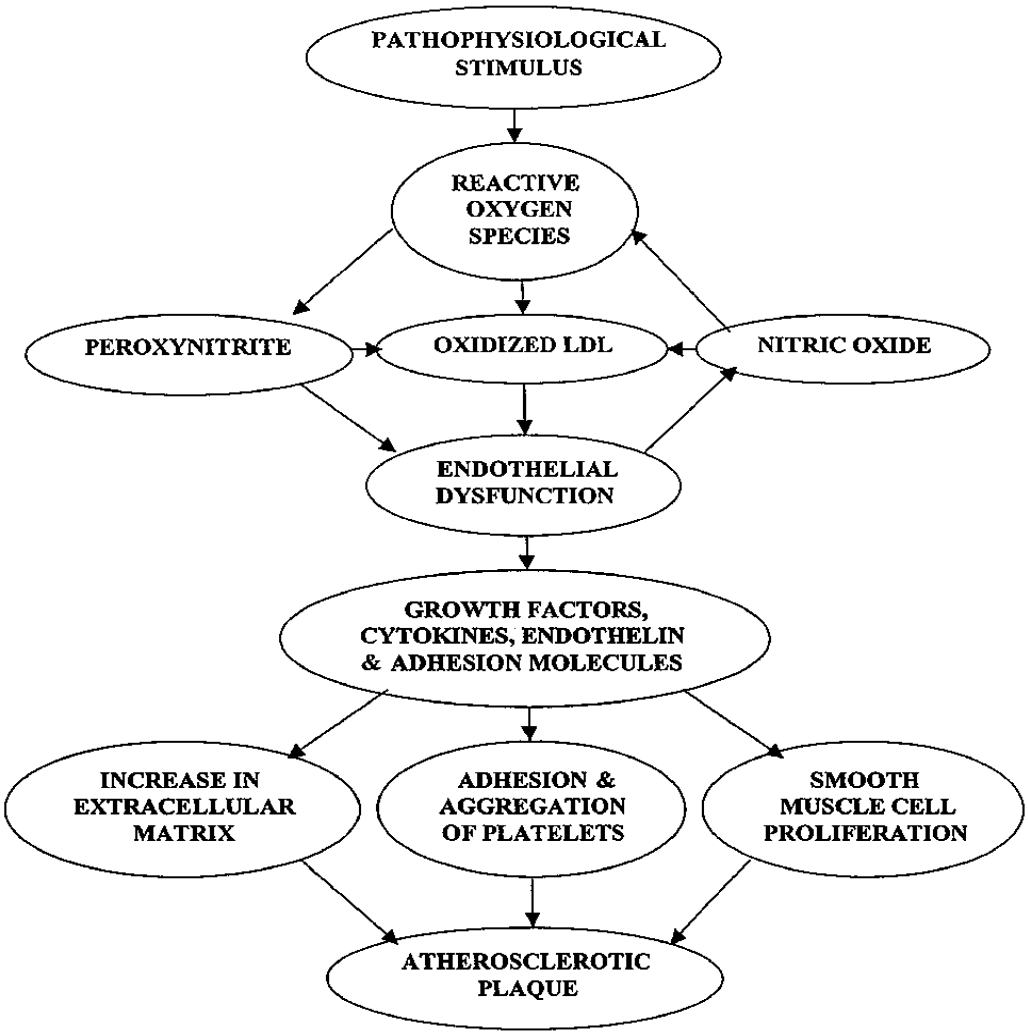
Endothelial injury plays a pivotal role in the pathogenesis of atherosclerosis. A plethora of factors such as excessive smoking, rise in cholesterol levels, chronic diseases like diabetes, hypertension, excessive physical activity and resistance to blood flow in the arteries initiates atherosclerosis by promoting endothelial dysfunction. These factors serve as stimuli for low density lipoprotein accumulation in the arterial vessels. ROS generated by a diversity of extracellular and intra-cellular mechanisms leads to oxidation of LDL.

Fig3.6: Pathogenesis of atherosclerosis



Source: Robbins textbook of basic pathology 8 th edition chapter 11 blood vessels,fig 11-9, page 499

Fig 3.7: Schematic representation of the involvement of oxidized LDL, endothelial cell injury and vascular smooth muscle cell proliferation in the development of Atherosclerotic plaque.



Source: Singh RB, Mengi SA, Y-J Xu, Arneja AS, Dhalla NS.

Pathogenesis of atherosclerosis: A multifactorial process.

Exp Clin Cardiol 2002; 7(1):40-53.

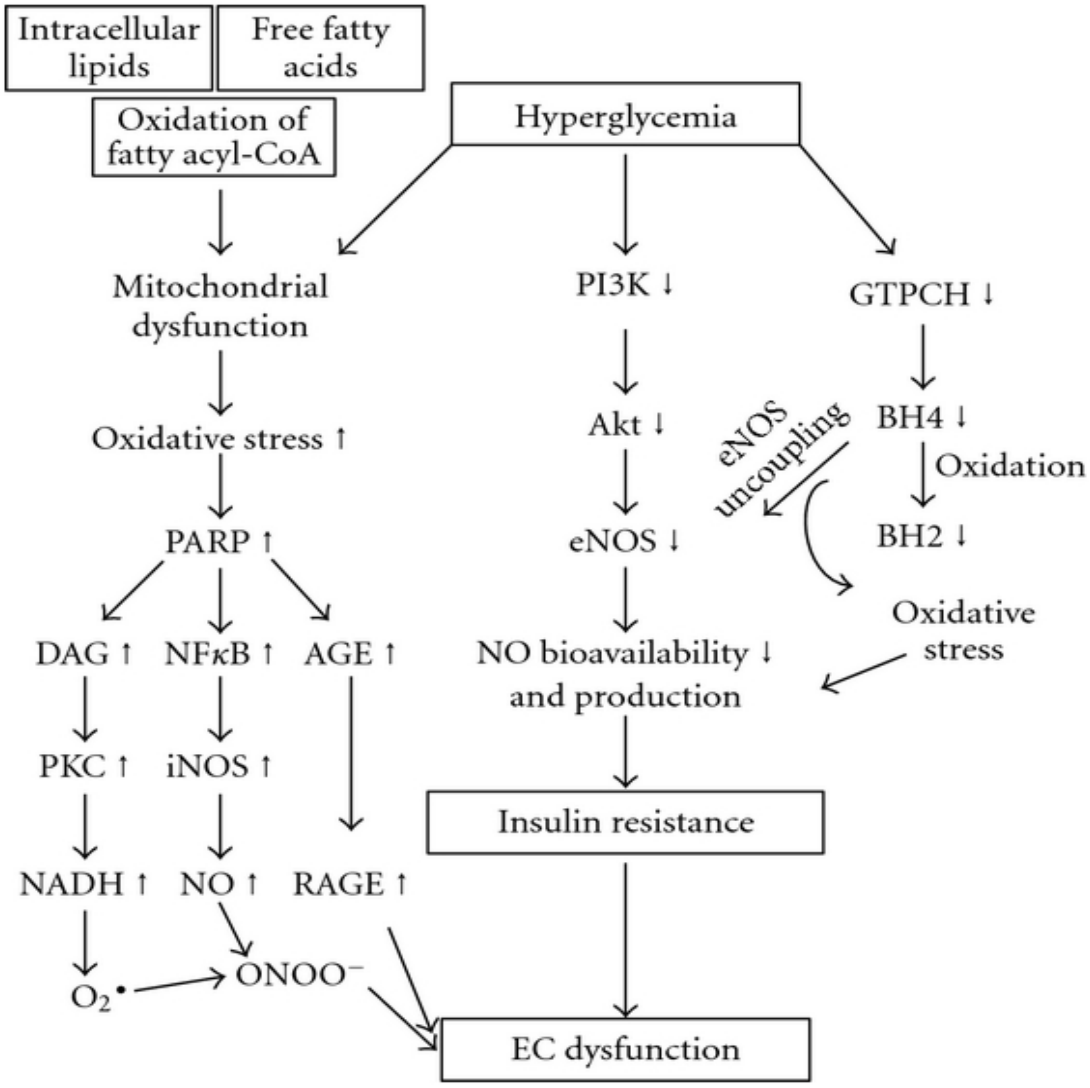
Oxidized LDL accelerates the recruitment and withholding of monocytes and macrophages. It also promotes the synthesis of cytokines and various other growth factors. They bind to scavenger receptor and activate endothelial cells, smooth muscle cells and monocytes. Oxidized LDL also mediate vasoconstriction, formation of thrombus and aggregation of platelets through the activation of protein kinases present inside the cell and transcription factors like NFκB. Oxidized LDL also stimulates the expression of cellular adhesion molecules on endothelial cells, macrophages and monocytes . It stimulates the production of various cytokines and growth factors in vascular smooth muscle cells for example monocyte chemoattractant protein-1, platelet-derived growth factor and procoagulant factors such as plasminogen activator inhibitor-I. Oxidized LDL produces vasoconstriction by decreasing the formation of the vasodilators such as nitric oxide and prostaglandin by the endothelium which enhances the synthesis of the vasoconstrictor endothelin-1. The uptake of oxidized LDL by monocyte-derived macrophages slows down macrophage migration in the subendothelial space and leads to the formation of foam cells which are the hallmark of atherosclerotic lesions[35].

Diabetes mellitus and cardiovascular disease:

Diabetes increases the risk of cardiovascular disease and coronary artery disease and is said to be the cause for about 75% of deaths in diabetics [36]. Incidence rate of myocardial infarction in diabetic individuals was similar to the incidence rate in non-diabetic individuals who had previous history of myocardial infarction. Recent studies have concluded that increased levels of C-reactive protein, fibrinogen and leukocytosis are the other risk factors for cardiovascular disease (CVD) in diabetic individuals [37].

Cardiovascular diseases are the most common cause of morbidity and mortality in people with type 2 diabetes mellitus[38]. Diabetes, a chronic disease is considered to be a state of low grade inflammation and immune activation leads to insulin resistance in pre-diabetic and diabetic individuals. This eventually increases the risk for cardiovascular diseases[39]. Patients with diabetes mellitus aggravate other co-morbidities like hypertension, obesity and altered lipid profile which in turn increase the risk for Cardio Vascular Disease [40]. National Cholesterol Education Program considers diabetes mellitus to be a risk factor for coronary heart disease [41].

Fig 3.8: Diabetes and Endothelial dysfunction



GTPCH: GTP cyclohydrolase; BH4: tetrahydrobiopterin;

BH2: dihydrobiopterin.

Source: Gopi Krishna Kolluru et al. Endothelial Dysfunction and Diabetes: Effects on Angiogenesis, Vascular Remodeling, and Wound Healing. International Journal of Vascular Medicine.Volume 2012, Article ID 918267

Glycemic control and CVD:

There were numerous studies depicting the significance of glycemic control in patients with diabetes and cardiovascular disease. The United Kingdom Prospective Diabetes Study (UKPDS), have shown that early intensive treatment of hyperglycemia in newly diagnosed Type 2 DM patients in the first five years of disease protects against cardiovascular diseases, when compared to patients in the conventional treatment group[42].

Veterans Affairs Diabetes Trial (VADT) was done in elderly diabetic patients. Mean duration of the disease in these patients was 10 years. When subjected to an intensive glycemic control, they had no protection against cardiovascular diseases. 40% of patients included in this study had previous history of cardiovascular disease[43].

Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was done with the aim of reducing HbA1c below 6% in Type 2 DM patient .According to this study intensive glycemic control had no effect on reducing cardiovascular events instead it increases body mass index,risk of hypoglycemia and also increases mortality[44].

Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial

was done with the aim of reducing HbA1c to 6.5% by treating them with oral hypoglycemic agents like gliclazide and other drugs. Similar to ACCORD trial,this study also had no effect on reducing cardiovascular events . But the incidence of diabetic nephropathy was reduced[45].

DCCT study showed findings quite different from ACCORD, ADVANCE trial. DCCT study was done in patients with short duration of diabetes who had no cardiovascular risks. The early initiation of treatment in these patients to reduce the HbA1c levels below 7% lowers the incidence of cardiovascular diseases. But this does not hold good for older patients with persistently high blood sugar and with increased risk for cardiovascular events[46]. This early protection is due to metabolic memory. Metabolic memory has a long term protective effect on target organs in later years due to intensive blood glucose control achieved in early years. The mechanisms behind metabolic memory appear to be due to oxidative imbalance, low level inflammation and endothelial dysfunction resulting from epigenetic and metabolic changes inside the cell [47].

German Diabetes Intervention Study was done in newly diagnosed type 2 diabetic patients. According to this study controlling post-prandial hyperglycemia in newly diagnosed patients had much more

benefit in reducing the incidence of CVD and overall mortality rather than controlling fasting blood glucose[48].

Role of inflammation in diabetes mellitus:

Inflammation is a short term protective tissue response elicited by the body to deal with injuries and microbial infections. Inflammation can be eminent in chronic diseases such as diabetes mellitus, chronic kidney disease and liver diseases. Abnormal levels of chemokines released by the expanding adipose tissue in obese individuals stimulates monocytes and thereby increases the synthesis of pro-inflammatory cytokines like interleukin 6,interleukin 1 β and tumor necrosis factor (TNF)-α. The secreted cytokines down regulate most important anabolic pathways concerned with insulin signaling and also mediates insulin resistance in peripheral tissues thus increasing the risk for Type 2DM. Apart from their effect on insulin resistance, they also exert an influence on hepatocytes and upregulates the synthesis of very-low density lipoproteins (VLDL), resulting in dyslipidemia. They also increase the synthesis of fibrinogen, an atherosclerotic risk factor secreted by the liver cells. Cytokines blocks the activation of the liver X receptors (LXR), leading to cholesterol accumulation which ultimately triggers the synthesis and secretion of acute-phase reactants like C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), serum amyloid-A, α1-

acid glycoprotein, and haptoglobin by hepatocytes. These are together referred to as inflammatory markers. Acute-phase reactants characterize the early stages of Type 2DM and their levels increase with the progression of disease and development of complications[49].

hsCRP- predictor of cardio vascular disease:

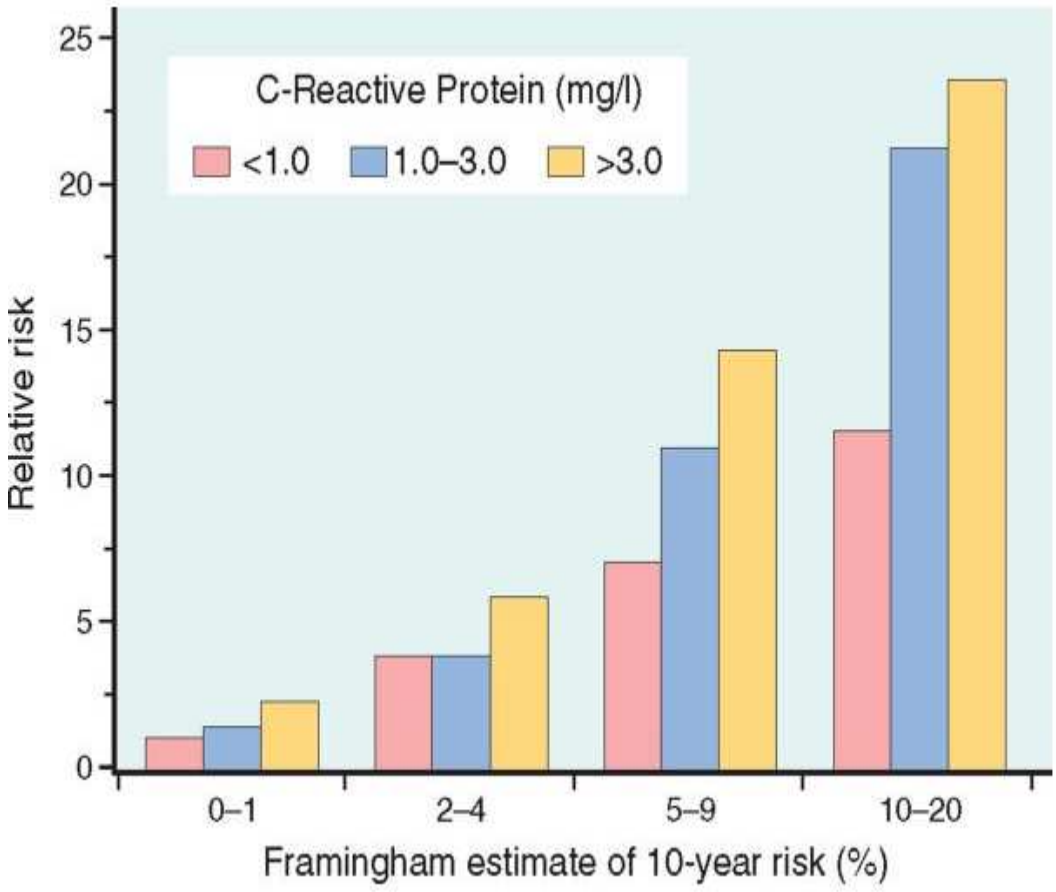
Nearly half of all myocardial infarction and stroke occurs without elevated cholesterol levels. So there is a need for a marker which has a critical role in inflammation and atherothrombosis. C-reactive protein is a multi-complex protein whose level increases when there are instances of inflammation in the body. CRP being an acute phase reactant may rise in infection and trauma also. Though it is an acute phase protein CRP is biologically stable over a longer period of time[50]. But studies have shown CRP, when measured with high-sensitivity assays appropriately in steady individuals, is more specific for predicting future cardiovascular events[51].

One of the previous studies has shown that highest quartile of hsCRP had two times the risk of future stroke (51.9 -Relative Risk; 95% Confidence Interval,1.1–3.3), thrice the risk of future myocardial infarction (52.9-Relative Risk; 95% Confidence Interval, 1.8–4.6) and the risk of future peripheral vascular disease is increased four times (54.1-Relative Risk; 95% Confidence Interval, 1.2–6.0)[52].Numerous studies

indicate that the relationship between hsCRP and future cardiovascular risk are largely independent of cholesterol and other risk factors[53, 54, 55].

The CDC-AHA"Workshop on Inflammatory Markers and Cardiovascular Disease: Application to Clinical and Public Health Practice" suggests that apart from all other markers of inflammation such as serum amyloid A, leukocyte count and fibrinogen , hs-CRP levels are more stable with high assay precision, accuracy and accessibility. American Heart association has set hsCRP cut points to be < 1 mg/L, 1 to 3 mg/L, and > 3mg/L which corresponds to low-risk, medium-risk, and high risk groups respectively. CRP levels serves as a prognostic marker when used along with LDL cholesterol level or Framingham risk score[56]. Type 2 diabetes patients with acute coronary syndrome, CRP serves as an independent marker for predicting cardiovascular death[57].

Fig 3.9: Framingham risk score for prediction of cardiovascular events based on hsCRP values



Source: Ridker PM et al: Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 2002;347:1557

Clinically hsCRP estimation should not be done when there is evidence of infection or trauma recently[50]. hsCRP measurement gives prognostic information at every level of metabolic syndrome[58].

hsCRP enhances atherosclerosis by the following mechanisms [59, 60].

- Activates complement cascade
- Expression of adhesion molecules like E-selectin and vascular cell adhesion molecule-1 is induced
- Expression and action of plasminogen activator inhibitor-1 in Human endothelial cells is increased
- T-cell-mediated endothelial cell destruction is enhanced
- Entry of LDL particles into macrophages is also enhanced

Oxidative stress in diabetes mellitus:

Oxidative stress occurs either due to increased synthesis of reactive oxidizing species or a marked reduction in the efficiency of antioxidant defense mechanism[61]. Oxidative damage to biological molecules like proteins, lipids, carbohydrates and nucleic acids has been concerned in the pathogenesis of many chronic diseases like diabetes mellitus, cardiovascular diseases and cancer.

Reactive Oxygen Species (ROS) comprises of a large number of reactive molecules and free radicals formed from molecular oxygen. ROS are synthesized as byproducts when electrons are transported in mitochondria during aerobic respiration. They may also be produced by oxidoreductase enzymes and during metal catalyzed oxidation reactions. ROS are necessary for maintaining redox potential for normal cell growth, gene expression and the activation of cell signaling pathways including apoptosis. But at high concentration ROS are able to cause oxidative damage[62].

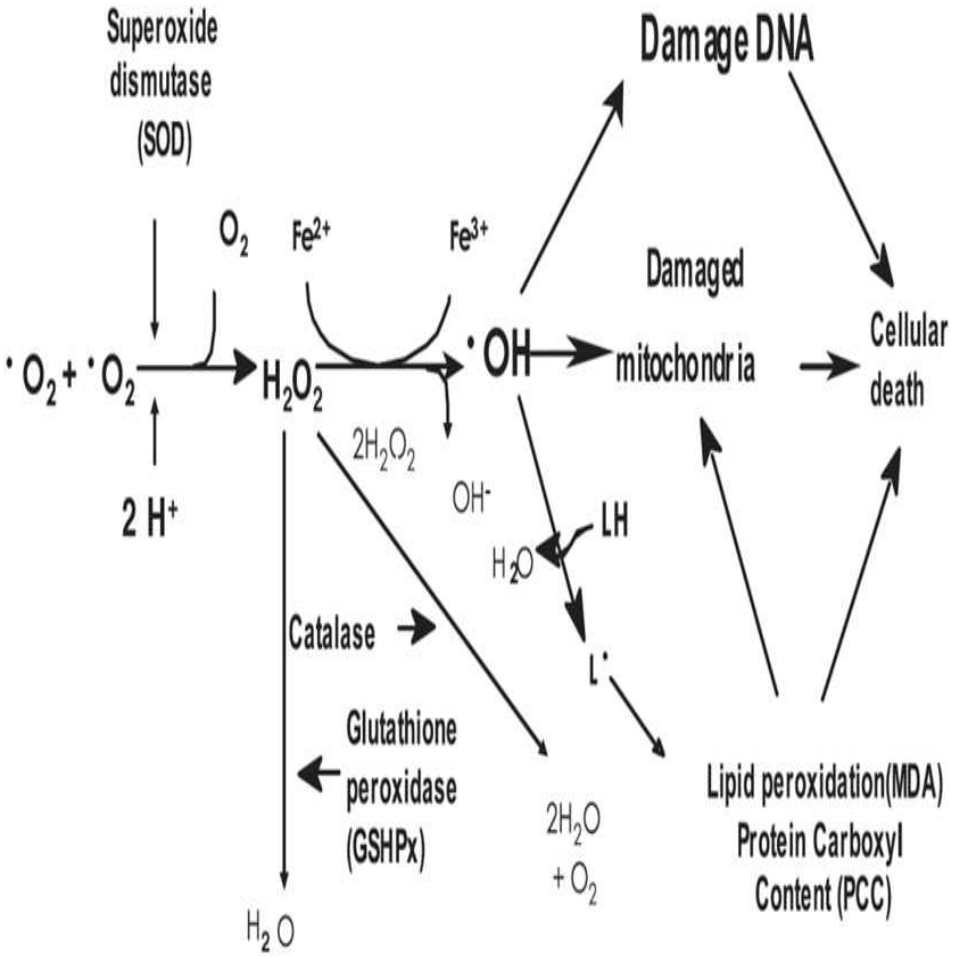
Radical is any atom or molecule with an unpaired electron in its outermost shell which makes it extremely reactive. The two unpaired electrons in the outermost electron shell of atomic oxygen in separate orbitals make it susceptible to radical formation. The antibonding orbitals of oxygen have the capacity to accept electrons which undergoes reduction during the process and thus enabling it to act as a strong oxidizing agent. Addition of electrons to oxygen sequentially results in the production of numerous ROS. The various ROS generated are superoxide radicals, H_2O_2, nitric oxide derivatives, hydroxyl radical and ion. Cells have a plethora of mechanisms to protect against the deleterious effects of ROS[63].

Addition of one electron to oxygen leads to the formation of superoxide anion $O_2^{-}\cdot$.Although a weak oxidizing agent $O_2^{-}\cdot$ serves as a source for the production of strong oxidising agents. $O_2^{-}\cdot$ in aqueous solution is short-lived due to the rapid dismutation of superoxide anion to hydrogen peroxide and O_2. Though H_2O_2 is not a radical it has the ability to be formed by reduction of Oxygen by two electrons. This reaction sequence is common to a number of flavoprotein oxidases[64]. Hydrogen peroxide is a weaker oxidizing agent than $O_2^{-}\cdot$ Unlike $O_2^{-}\cdot$, H_2O_2 freely diffuses across biological membrane and is more stable than superoxide anion. H_2O_2 in the presence of transition metals such as iron, copper forms the hydroxyl radical (OH·) which is highly reactive and the hydroxide ion[65].

The hydroxyl radical being very reactive removes electrons from any molecule along its path thereby converting it into a free radical. This initiates a chain of reactions by the free radicals. Lipids in cell membrane are more prone to damage by these radical chain reactions[63].It can also start lipid peroxidation by taking an electron from polyunsaturated fatty acids.H_2O_2 is converted to hypochlorous acid (HOCl) in the presence of chloride ion. HOCl is highly oxidative and plays an important role in killing pathogens in airways .Moreover it also induces DNA–protein interactions and produce pyrimidine oxidation products and add chloride

to DNA bases[66].Peroxyl radical ($ROO^{\cdot}$) initiates a chain reaction and transforms polyunsaturated fatty acids into lipid hydroperoxides which are unstable and easily decompose to secondary products,such as aldehydes such as 4-hydroxy-2,3-nonenal and malondialdehydes (MDAs).Lipid peroxidation disturbs the integrity of the cell membrane[67].

Fig 3.10: Mechanisms of cellular oxidative damage



Sources of ROS

There are two major sources of ROS namely enzymatic and nonenzymatic sources. Among the sources,under reducing conditions mitochondria accounts for 1–2% of total Oxygen consumption. Superoxide dismutase (SOD) when present in high concentration maintain the superoxide ion levels inside the mitochondria at a very low and steady level. Mitochondria also functions as an Oxygen sensor to mediate hypoxia-induced gene transcription. Apoptosis induced by tumor necrosis factor (TNF)-α and interleukin (IL)-1 involves ROS derived from mitochondria. Endoplasmic reticulum derived oxidants and growth factor signaling regulates protein folding and secretion.

Electrons leaking from electron transport systems generate ROS which has the ability to damage cellular DNA. Glycolate oxidase, D-amino acid oxidase, urate oxidase, L-alpha-hydroxyacid oxidase and peroxisomal fatty acyl-CoA oxidase are the major source of total cellular H_2O_2 production. In addition to oxidases present in the membrane of the cell, enzymes like xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase are also capable of generating ROS during catalytic process.

Dopamine, epinephrine, flavins and hydroquinones on auto-oxidation form the major source of ROS production within the cell [65, 68]. Phagocytic cells on stimulation produce ROS. This was referred to as respiratory burst which is due to the increased utilization of oxygen by these cells. This reaction is mediated by the multicomponent enzyme which is membrane bound namely NADPH oxidase.

Antioxidants:

Antioxidants are substances that have the ability to deactivate and scavenge the free radicals before they induce cell damage. They are necessary for maintaining normal cell growth and well-being.

Antioxidants can be derived from nutrient sources. They include water soluble vitamin such as vitamin C, fat soluble vitamin such as vitamin E and its derivatives (tocopherols and tocotrienols), precursors of vitamin A (carotenoids) and other compounds such as glutathione and lipoic acid. The antioxidant effect of vitamin C begins before the initiation of lipid peroxidation. Fatty acids present in membranes are protected from lipid peroxidation by vitamin E. Beta carotene and other carotenoids work synergistically with vitamin E and protect lipid-rich tissues[69].

Lipoic acid functions as universal antioxidant by quenching free radicals in both lipid and aqueous domains[70]. Antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase are called as primary antioxidant enzymes. Superoxide dismutase catalyzes the conversion of two superoxide anion to form Hydrogen peroxide and molecular oxygen. In this reaction one O_2^- is oxidized to form molecular oxygen and the other O_2^- is reduced to form H_2O_2.Glutathione peroxidase catalyses the reduction of H_2O_2 to water which requires two reduced glutathione molecules (GSH). GSH is a tripeptide containing amino acids cysteine, glutamic acid and glycine. Moreover sulfhydryl groups such as thiol and small tripeptide glutathione act as non enzymatic low molecular weight antioxidants[71].

Oxidative stress in DM:

Increased glucose levels leads to increased production of superoxide radicals by mitochondria. These radicals bring about DNA damage which increases the activity of poly-ADP-ribose polymerase-1 (PARP-1). This enzyme plays a major role in DNA repair mechanisms and pathways related to apoptosis. PARP-1 activation results in inhibition of glyderaldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis by poly-ADP-ribosylation. This leads to an increase in the intermediates of glycolytic pathway upstream of GAPDH.

As a result, two major pathways related to diabetic complications namely advanced glycation end product pathway (AGE) and diacyl glycerol-protein kinase C (DAG-PKC) pathway are activated. Activation of the AGE pathway results in nonenzymatic synthesis of methylglyoxal. Increase in fructose 6-phosphate increases the flux through the hexosamine pathway and leads to a rise in blood glucose levels. The increase in glucose is shunted through the polyol pathway, where NADPH is consumed. As a result glucose is reduced to sorbitol by aldose reductase. Synthesis of reduced glutathione, a powerful antioxidant requires the cofactor,NADPH .Depletion of NADPH, modifies the redox state of the cells exacerbating intracellular oxidative stress by scavenging ROS. Further, sorbitol on oxidation forms fructose by the action of the enzyme sorbitol dehydrogenase. The resulting increase in NADH/NAD+ ratio activates protein kinase C pathway.

When there is a rise in intracellular glucose level, excess fructose-6-phosphate is transformed to UDP-N Acetylglucosamine, which plays a essential role in the production of carbohydrate chains of proteins and lipids. UDP-N Acetylglucosamine is also needed for the post-translational modification of proteins present in the cytoplasm and nucleus in serine and threonine residuesre. Increase in dihydroxyacetone phosphate in hyperglycemic environment leads to increased denovo

synthesis of DAG which activates protein kinase C isoforms. PKC isoforms stimulates a large range of cellular signals that activates NADPH oxidase and nuclear factor -κB leading to disproportionate reactive oxygen species production.

Apart from the activation of AGE production and the PKC pathway, glyceraldehyde 3-phosphate can undergo autooxidation generating H_2O_2 which further contributes to oxidative stress[72].

The glucotoxicity in diabetic patients leads to the formation of intracellular and extracellular AGEs. Glucose undergoes auto-oxidation to glyoxal. Amadori product on decomposition forms 3-deoxyglucosone. Methylglyoxal is formed by nonenzymatic phosphate elimination from glyceraldehyde phosphate and dihydroxyacetone phosphate which on reacting with amino groups of intracellular and extracellular proteins forms AGE products. The mechanisms by which AGE precursors causes cell damage are by altering the function of intracellular proteins,modification of extracellular matrix components. These modified proteins bind to AGE receptors on macrophages, monocytes, endothelial and smooth muscle cells present on the vessels and induce the formation of ROS, leading to the activation of PKC[72].

These highly reactive oxygen species have the capacity to alter the function of nucleic acids and cause cell damage by modifying carbohydrates, proteins and lipids.

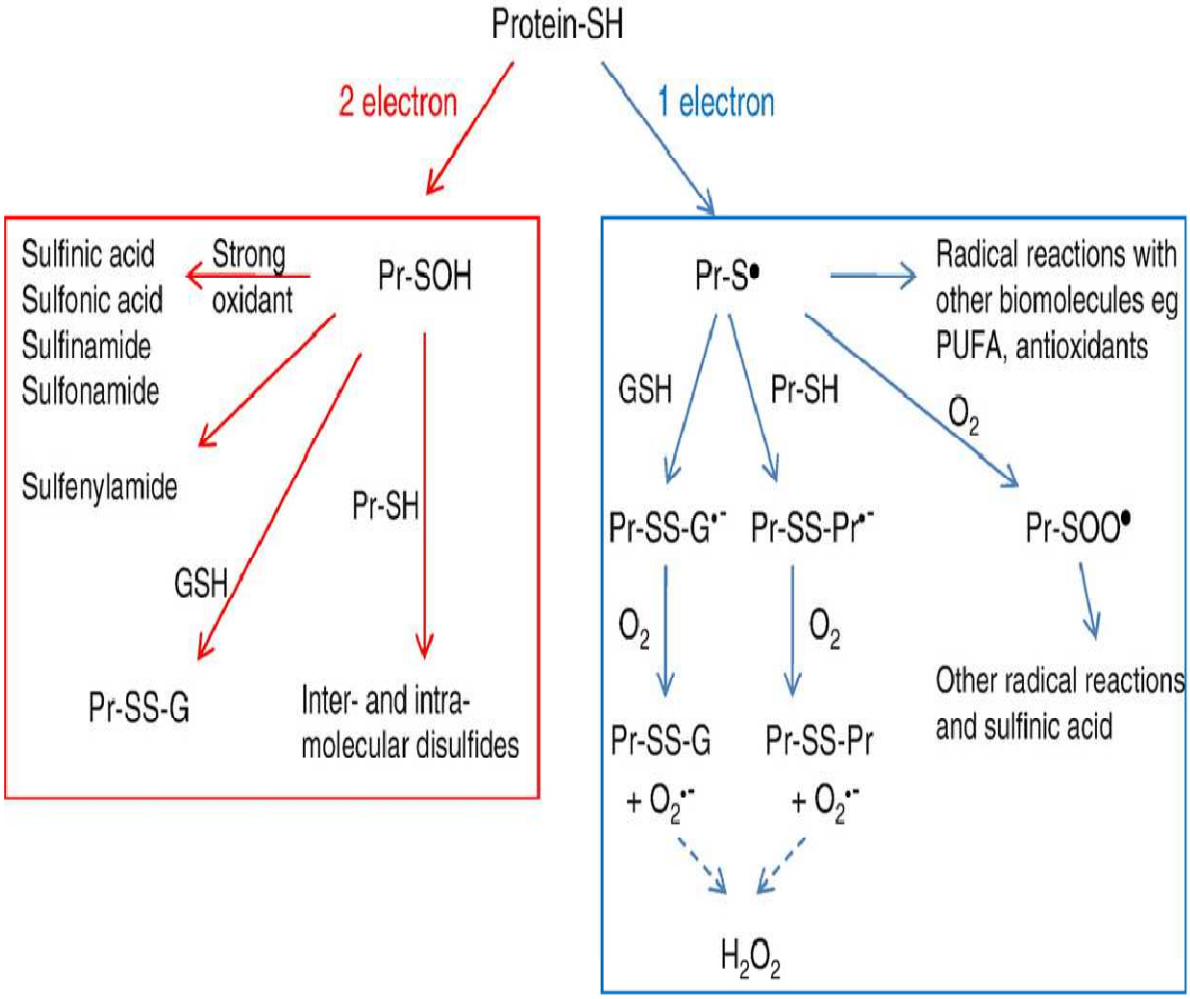
Protein thiols, marker of oxidative stress:

Thiols are organosulfur compounds containing alcohol as a functional group. They encompasses the sulfhydryl groups and disulphides present on proteins with homocysteine,glycine, cysteinylglycine and glutathione. They are the major antioxidants in the body in defense against reactive oxygen species. Oxidation of thiols can lead to the formation of intra molecular disulfides, intermolecular mixed disulfides, mixed disulfides formed between protein thiols and GSH[73].The sulfhydryl group present in cysteine is essential for the normal biological activity of glutathione. Oxidation of thiols can also result in damage to proteins with severe functional consequences.

There are two major proposed mechanisms for redox signaling. One is based on thermodynamic principles which states that all thiols and disulfides exist in equilibrium with each other.GSH is a redox buffer and exists in its oxidised or reduced form based on the redox potential of the cell.

The other mechanism is based on the kinetic properties of specific and sensitive targets. According to this mechanism, nearly all physiological oxidants react with thiols although most show selectivity for thiolate anion by causing one electron or two electron oxidations. The one electron oxidation forms a thiyl radical, which undergoes further reactions including transfer of radicals to the antioxidant ascorbate. The thiyl radical preferentially reacts with a thiolate anion from a protein or glutathione to form the disulfide anion radical. The two electron oxidation of thiols initially produces a series of intermadiates like sulfenic acid, sulfonamide, sulfonamide, sulfenylamide, mixed disulfides, inter and intra molecular disulfides (fig 3.10)

Fig 3.11: The one and two electron oxidations of protein thiols



Adapted from Winterbourn CC, Hampton MB. Thiol chemistry and specificity in redox signaling Free Radic Biol Med. 2008 Sep 1; 45(5):549-61.

Almost all physiologically relevant oxidants are capable of reacting with thiols. H_2O_2 reacts directly with thiolate anions, following the two electron oxidation path. Radical oxidation reactions can produce thiyl and sulfinyl radicals during the process of a radical chain reaction. Other common thiol oxidants like peroxynitrite, can react directly with thiols or break down into the reactive hydroxyl radical, nitrogen dioxide and hypohalous acids. Thus the cells respond to oxidative stress by altering the redox state of critical thiols[74].In human plasma protein sulfhydryl groups is in the concentration of 0.4–0.5 mM , where as low-molecular mass thiols is in the concentration of 0.1–20 μM [75]. Albumin being the most abundant protein in plasma, total thiol level in the body depends on the amount of thiol groups present on albumin.

Thiol groups present on amino acids like cysteine stabilize protein structures by forming covalent disulfide bonds and by their high reactivity and redox properties[76]. Modification of thiol groups results in the formation of mixed and internal disulfides, thiyl radicals, sulfinic and sulfonic acids. Protein sulfhydryls on oxidation forms mixed disulfides (protein S thiolation) and on reduction once again forms sulfhydryls (dethiolation) .This is considered to be the early cellular response against oxidative stress[73].Protein thiols have the ability to scavenge two third of the total reactive species generated in the body. Thus the level of protein

thiols in the serum indicates the antioxidant level in the body. Increase in lipid peroxidation and protein oxidation causes a fall in protein thiol levels in the serum[77].

Glutathione and thioredoxin are thiol buffers present inside the cell. The balance between the levels of these intracellular thiol buffers and the reactive oxygen species determines the redox state of the cell. When there is a remarkable rise in reactive oxygen species than that of the compensatory endogenous thiol buffers, there is a persistent activation of genes and signaling pathways that stimulates apoptosis in the affected cells. Glutathione comprising of glycine, glutamic acid and cysteine is the most common nonprotein sulfhydryl compound and this forms the majority of endogenous thiol buffers in the cell[78].

Serum protein thiol levels are decreased in both type 1 and type 2 diabetes mellitus. This decrease was expounded to some extent by metabolic, inflammatory and iron alterations[79]. Serum protein thiols are also decreased in type 2 diabetes mellitus patients with complications[80].

4. Materials and Methods:

This study was conducted in the department of Biochemistry, PSG IMS&R during the period of june 2013 to june 2014 with the approval of institutional human ethics committee.

It is a cross sectional study including two groups:

Group 1: Type 2 diabetes mellitus patients without complications

Group 2: Type 2 diabetes mellitus patients with cardiac complications

Study was initiated after obtaining informed consent from the participants of the study.

Inclusion criteria:

For groups 1 & 2

- ✓ Age 30-75 years
- ✓ Males and females included
- ✓ Duration of diabetes: a minimum period of 6 months from the date of diagnosis

For group 1

- ✓ Type 2 Diabetic patients reporting to Endocrinology department and on treatment with Antidiabetic drugs

For group 2

- ✓ Type 2 Diabetic patients reporting to the department of Cardiology with cardiac complications and on treatment for the same

Exclusion criteria:

Subjects (all groups)

- ✓ With Hypertension
- ✓ With acute inflammatory diseases (trauma, viral and bacterial infections)
- ✓ With chronic inflammatory diseases (Rheumatoid arthritis, Systemic lupus erythematosus, Bronchial asthma, Tuberculosis, Sarcoidosis and any chronic granulomatous diseases)
- ✓ With malignant neoplasms
- ✓ With Creatinine clearance less than 15 ml/min/End Stage Renal Disease /Stage V of Chronic Kidney Disease.
- ✓ With visual defects and/or motor/sensory defects
- ✓ Subjects on treatment for the above mentioned conditions
- ✓ Subjects on hormone replacement therapy

Sample collection:

5 ml of random venous blood sample was collected in EDTA and heparin tubes. HbA1c, Protein thiol levels were analysed on the day of collection. Samples for hsCRP analysis were stored at -23˚c and were estimated collectively.

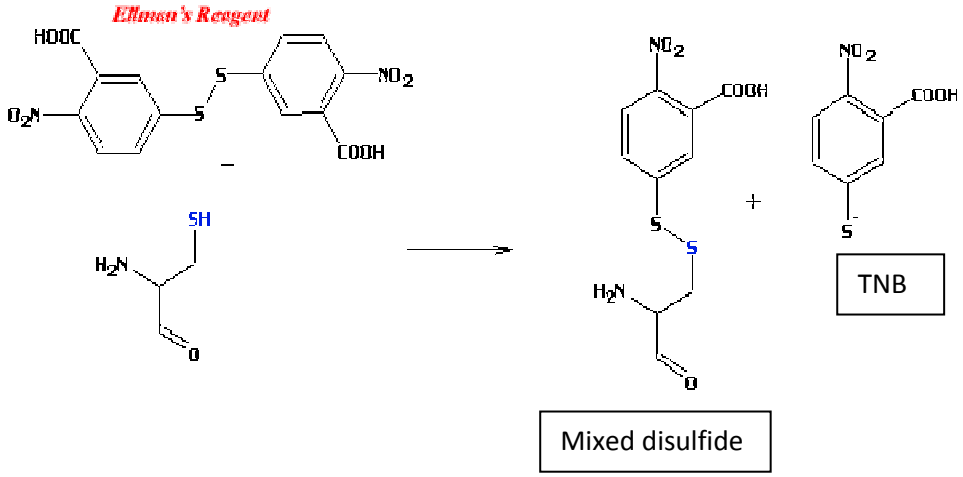
Assay of plasma protein thiols:

Method: Dinitrobenzene (DTNB)-Ellman's method using sphectophotometry[81, 82]

Principle:

5, 5'-dithiobis (2-nitrobenzoate) [DTNB] is a symmetrical aryl disulfide which in the presence of a free thiol group readily undergoes the thiol-disulfide interchange reaction. The TNB di-anion absorbs light intensively at 412nm than that of both disulfides. Since the stoichiometry of protein thiol to TNB synthesized is 1:1, number of thiol groups present can be assessed by the number of TNB formed. When denaturants are not present, only accessible thiols can react.The total number of reduced cysteine residues present can only be measured in the presence of chaotropic agents. Reduction of the protein followed by treatment with chaotropes and DTNB can only give the total number of cysteines (Cys-SH plus Cys-S-S-Cys). The total plasma protein thiol concentration was

measured using the molar extinction coefficient of TNB complex (13,600/M/cm) in the assay mixture at 412 nm.



Reagents:

1. Buffer: 0.2 M Na_2HPO_4 containing 2 mM Na_2 –EDTA, pH: 7.4:

   0.2M Na_2HPO_4 (MW: 142):

   2.84 gm of Na_2HPO_4 dissolved in 90 ml of Distilled water and made upto 100 ml with distilled water. Stored in amber color bottle at 4^oC.

Working Buffer:

Dissolve 74.4 mg of Na_2–EDTA (MW: 372.24) in 90 ml of 0.2 M Na_2 HPO_4 and made upto100 ml with 0.2 M Na_2 HPO_4.Prepared fresh before assay.

2. Phosphate Buffer saline (PBS) pH: 7.4:

0.9 gram of sodium chloride.

0.2302 gram of Disodium Hydrogen Phosphate (Na_2HPO_4)

0.0194 grams of Sodium Dihydrogen Phostphate (NaH_2PO_4);

All the above ingredients are dissolved separately in 80ml of distilled water. Mixed and then volume is made upto 100 ml with distilled water. Stored at room temperature.

3. 10mM of 5, 5'-Dithio-bis-(2-nitrobenzoic acid) in 0.2 Na_2HPO_4

(MW: 396.35):

Prepare 39.63 mg/10 ml in 0.2M Na_2 HPO_4.Store in amber color bottle at 4^oC. It is stable for 4 weeks if stored in dark bottles.

Test Procedure:

Dilution of plasma: 1:3; 100µl Plasma + 200 µl PBS.

PBS, DTNB, 0.2 M $Na_2 HPO_4$ in 2 mM EDTA were pre-incubated at 37^{o}C before the assay. Three Eppendorf tubes were labeled as Reagent Blank (RB), Test (T) and Sample Blank (SB).Pipetting was done as mentioned below:

REAGENT	Reagent Blank	Sample Blank	Test
0.2M,Na_2HPO_4 Containing 2mMEDTA, (µL)	900	920	900
PBS (µL)	100	-	-
10mM DTNB(µL)	20	-	20
Sample (µL)	-	100	100

The contents in each of the tubes were mixed in a Vortex, incubated 5min at room temperature.

- 2 min gap was left between Reagent Blank and Test and each was read after 5min.
- The solution was shifted to a cuvette and absorbance was estimated at 412 nm.
- The reaction takes about 5 minutes to go to completion, during which there was an increase in the absorbance. The maximum value was recorded.
- The sample blank was measured as bilirubin, beta carotene and other plasma constituents that absorb at 412 nm can interfere with protein thiol measurement

Calculations:

The absorbances for sample and reagent blanks were subtracted from test absorbance values to obtain the correct values.

$$X= T-(SB+RB) = \text{Abs. at } 412nm$$

Applying the molar extinction coefficient, absorbance at 412 nm was divided by 13,600/M/cm to get the molarity in the assay

Thiol concentration

$$\mu M/L = \frac{(\text{Tot.vol/sample.vol}) \times \text{dilution factor} \times \text{Abs at 412 nm}}{13600}$$

= 10.2 x 3 x Abs. at 412nm / 13600 M/L.

= 0.00225 x Abs. at 412 nm x 10^6 µMol/L.

= 2250 x Abs. at 412 nm, µMol/L

Linearity of the assay:

As per the literature, the linear range of the procedure is 35-1200 µMol in plasma with a coefficient of variation being 2.1%.

Assay of high sensitivity C reactive protein:

Method: Particle enhanced turbidimetric assay[83]

Principle:

Human C reactive protein agglutinates with monoclonal anti-CRP antibodies coated on latex particles. The precipitate is measured turbidimetrically at 552 nm

Sample: plasma.

Samples should be collected in Li- heparin containing tubes

Reagents:

Reagent 1: Bovine serum albumin and immunoglobulins (mouse) in TRIS buffer

Reagent 2= Standard Reagent: Glycine buffer with latex particles coated with anti CRP (mouse)

Test definition:

Mode of Measurement	Absorbance
Mode of Abs. calculation	Kinetic
Direction of reaction	Increase
Wavelength A/B	552nm
range of measurement	0.1-20
Unit of measurement	mg/L

In normal adults normal reference limit is < 5 mg/ L

For risk stratification

hsCRP value	Risk
< 1 mg/L	Low risk
1 to 3 mg/L	Medium risk
> 3mg/L	High risk

Pipetting parameters:

REAGENT	Volume	Diluents (H_2O)
Reagent 1	82 µL	48 µL
Sample	6 µL	-
Reagent 2	28 µL	14 µL
Total volume	178 µL	-

Measured with cobas 400 autoanalyser

Assay of Glycated haemoglobin: (HbA1c)

Method: Turbidimetric inhibition immunoassay[84]

Principle:

EDTA tube collected whole blood specimen is hemolysed after collection. Total hemoglobin and HbA_1c concentrations are determined. Total hemoglobin is measured colorimetrically. The percentage of HbA_1c is determined immunoturbidimetrically. The ratio of both these levels gives the final percent HbA_1c result.

EDTA collected blood is hemolysed with hemolysis reagent in the predilution cuvette. This leads to reduction in osmotic pressure which lyses the erythrocytes. Lysis of erythrocytes releases the haemoglobin. It is degraded by the proteolytic action of the enzyme pepsin. This renders the beta-N terminal structures more available for the immunoassay.

In the hemolysate, a colorimetric method helps in the determination of total haemoglobin. This is done on the basis of production of a brownish-green chromophore. This occurs in alkaline detergent solution using a cyanide free method.

The intensity of colour formed is directly proportional to the total hemoglobin concentration present in the sample. The concentration is

determined by sensing the increase in absorbance at 552nm. A fixed factor that is obtained from the primary calibrator chlorohemin calculates the test results.

Turbidimetric method to measure HbA_1c is done using monoclonal antibodies which are present attached to latex particles. The monoclonal antibodies bind the amino terminal fragments of HbA_1c. The unbound free antibodies agglutinate with an artificial polymer. The change in turbidity is inversely related to the quantity of bound glycated proteins. This is measured turbidimetrically at 552nm.

Pippetting parameters for measuring Hb:

REAGENT	Volume	Diluents (H_2O)
Reagent 1	120 µL	-
Sample	6 µL	0
Total volume	126 µL	-

Pipetting parameters for measuring HbA1c:

REAGENT	Volume	Diluents (H_2O)
Reagent 1	120 µL	-
Sample	6 µL	0
Standard reagent	24 µL	0
Total volume	150 µL	-

The measuring range for HbA1c lies between 0.3– 2.6 g/dL. This corresponds to a measuring range of 2-17% HbA1c at a typical hemoglobin concentration of 15 g/dL.

The end result of HbA1c is expressed in percentage and is calculated from HbA1c/Hb ratio.

According to IFCC

- HbA1c(%) = (HbA1c/Hb)*100

5. Statistical analysis:

The data obtained after estimation of plasma total thiols, hsCRP and HbA1c in two groups were statistically analyzed using SPSS software version 19.

Independent t test was used to analyze plasma total thiols, hsCRP and HbA1c levels between the two study population.

Pearson correlation coefficient was used to find out the correlation existing between plasma total thiols and hsCRP with HbA1c.

Since plasma total thiols and hsCRP levels were not normally distributed, Spearman`s rank correlation coefficient was done.

6. Results:

Fig 6.1: Age distribution among study groups

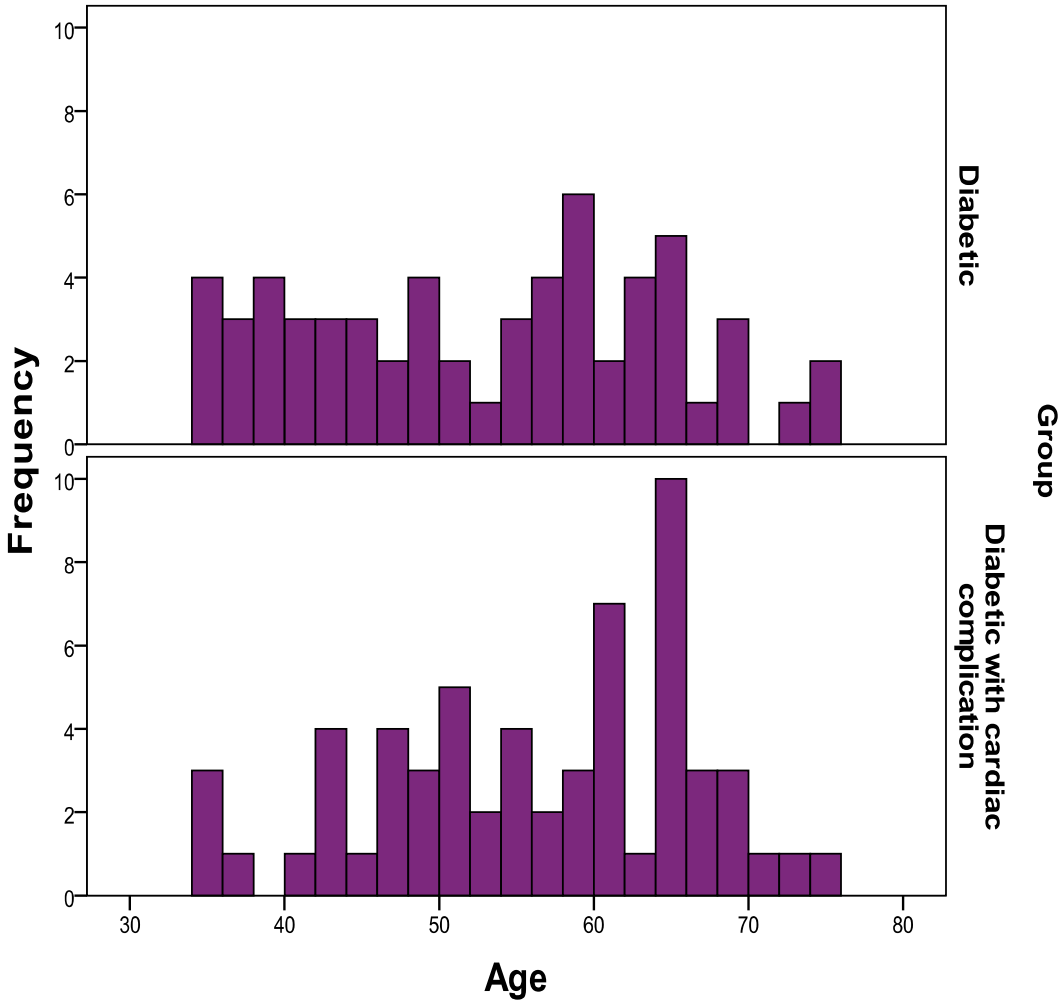


TABLE 1: MEAN AGE OF STUDY POPULATION

AGE IN YEARS		
	Diabetic patients	Diabetic patients with cardiac complications
MEAN±SD	52.57±11.51	55.70±10.12
p value	0.116 (Not significant)	

Fig 6.2: Sex distribution among study groups

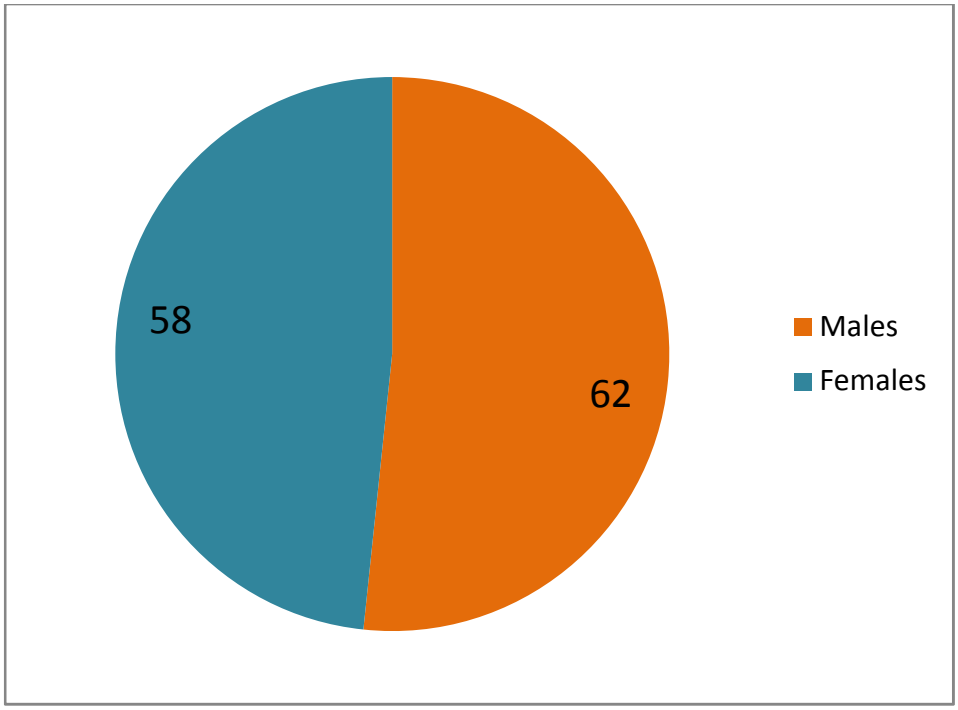
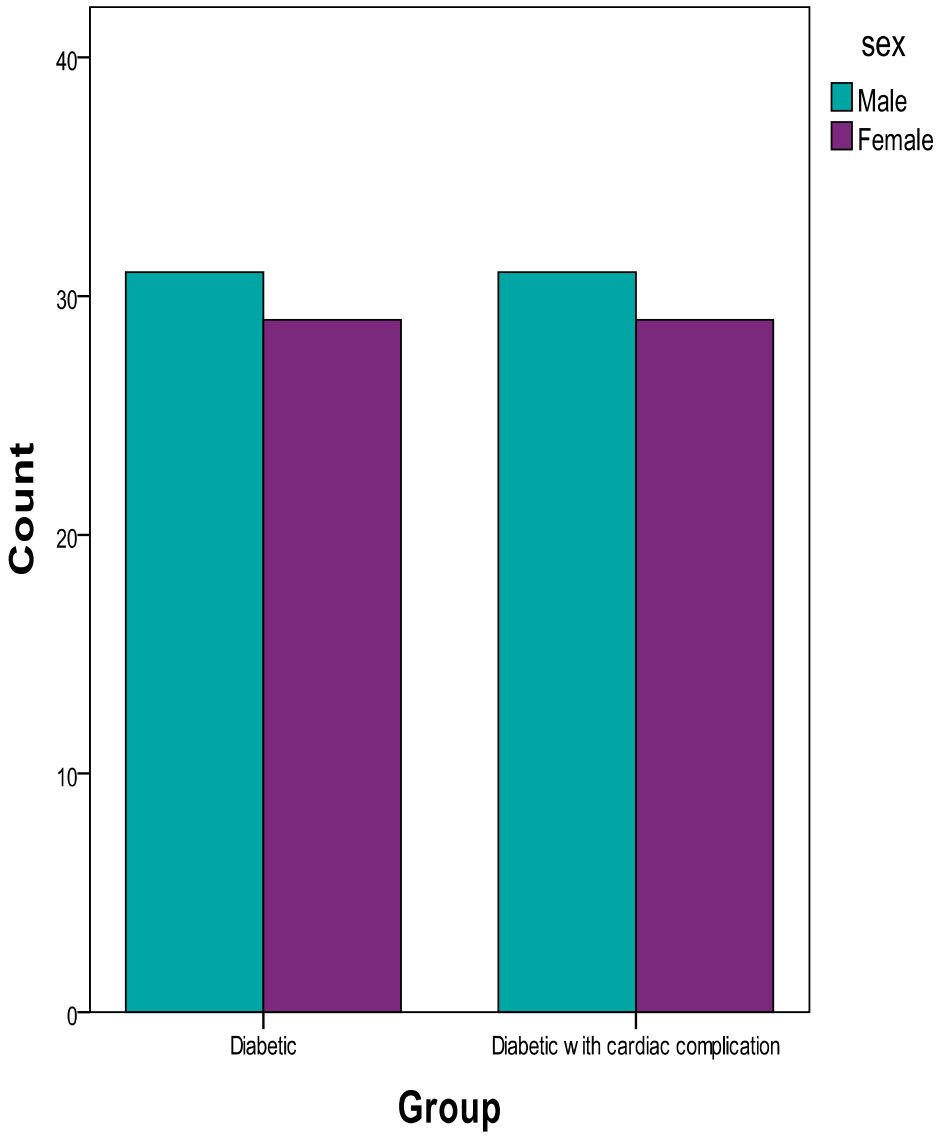


Fig 6.3: Sex distribution among study group



According to chi-square test, p value was found to be 0.715 (Not significant)

Fig 6.4: Duration of diabetes among study population

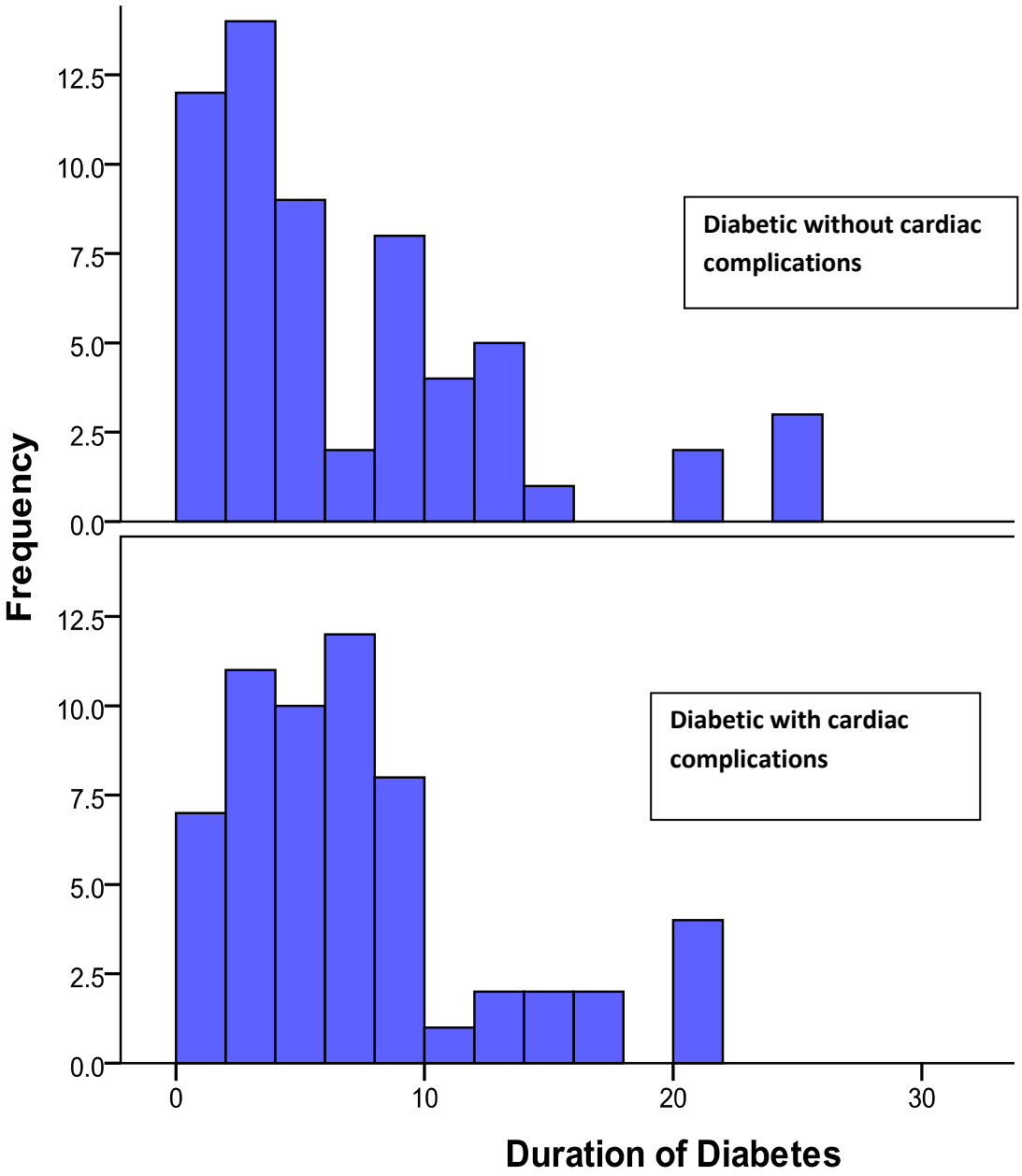


Fig 6.5: HbA1c levels in study group

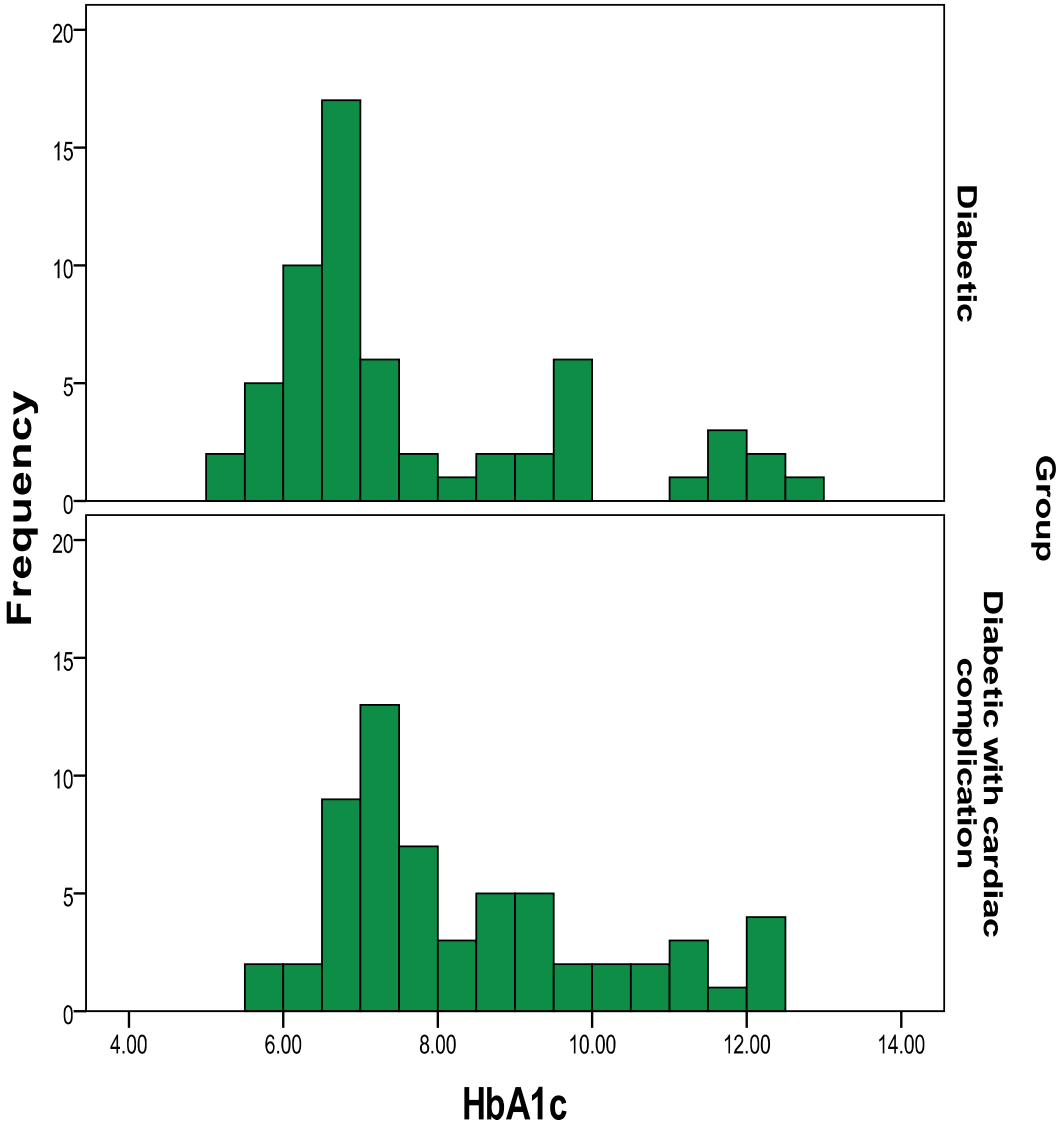
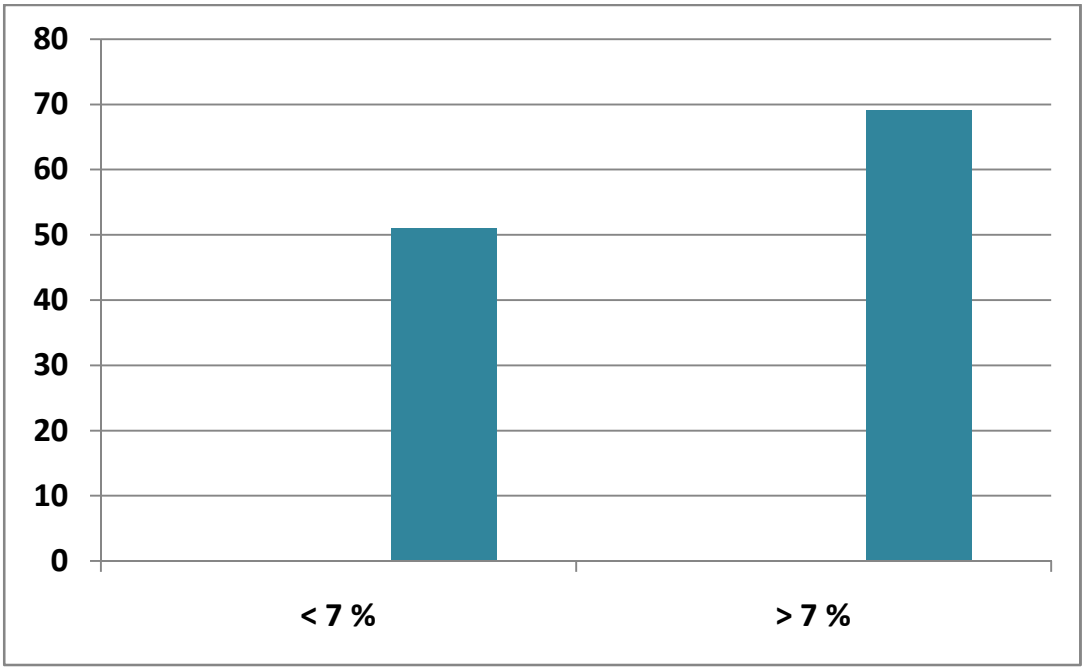


Fig 6.6: Frequency distribution of glycemic control in the study population



< 7 % is good glycemic control (51)

> 7 % is poor glycemic control (69)

Fig 6.7: hsCRP levels in study population

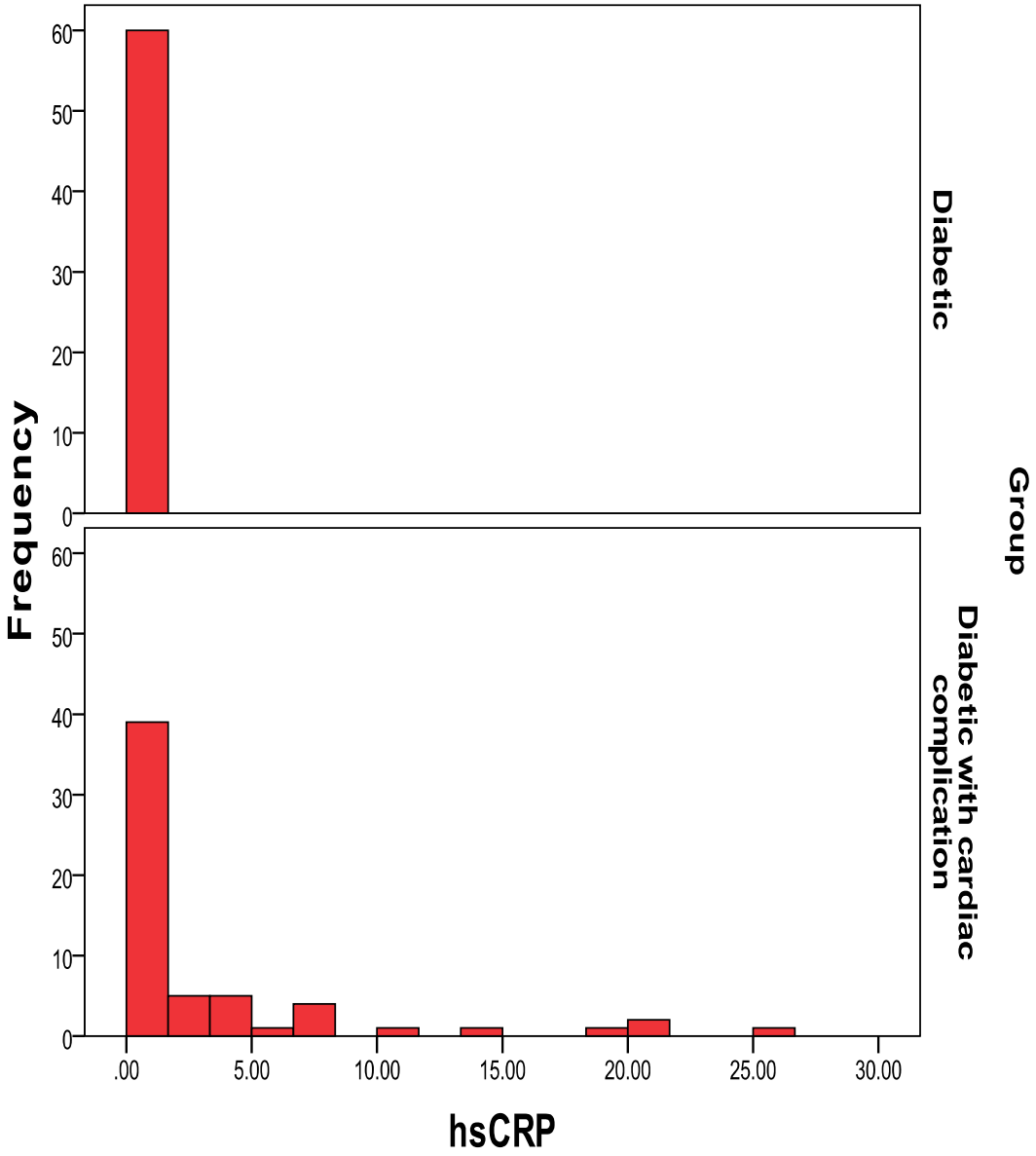
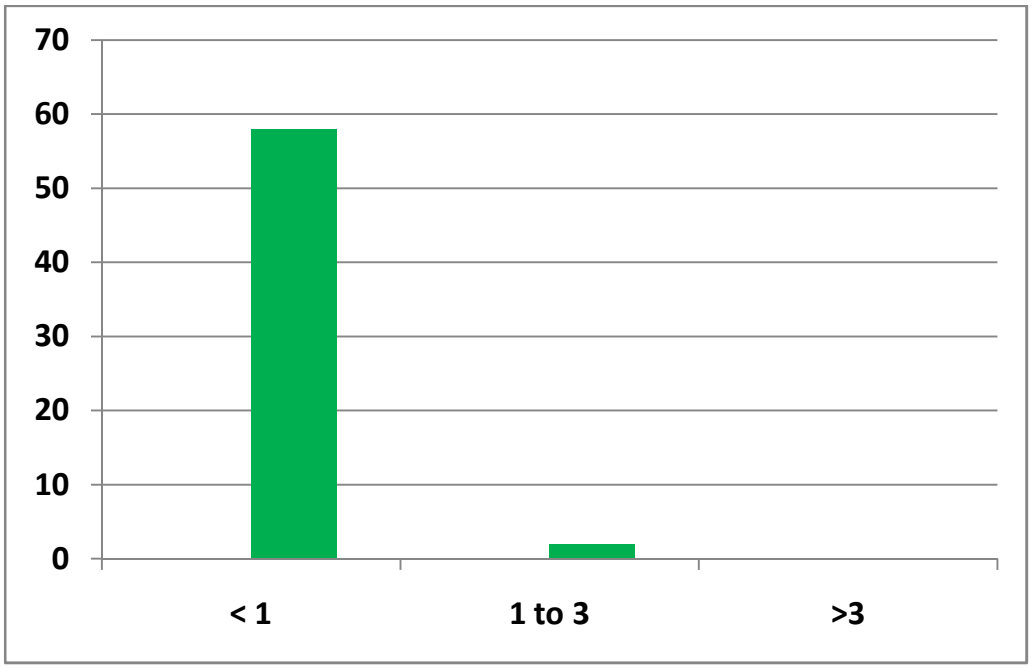


Fig 6.8: Frequency distribution of hsCRP levels in type 2 diabetic patients without complications based on Framinghams risk score



Risk stratification for cardiovascular events

< 1 mg/L – Low risk

1 – 3 mg/L – Medium risk

>3 mg/L - High risk

Fig 6.9: Levels of total plasma thiols in study population

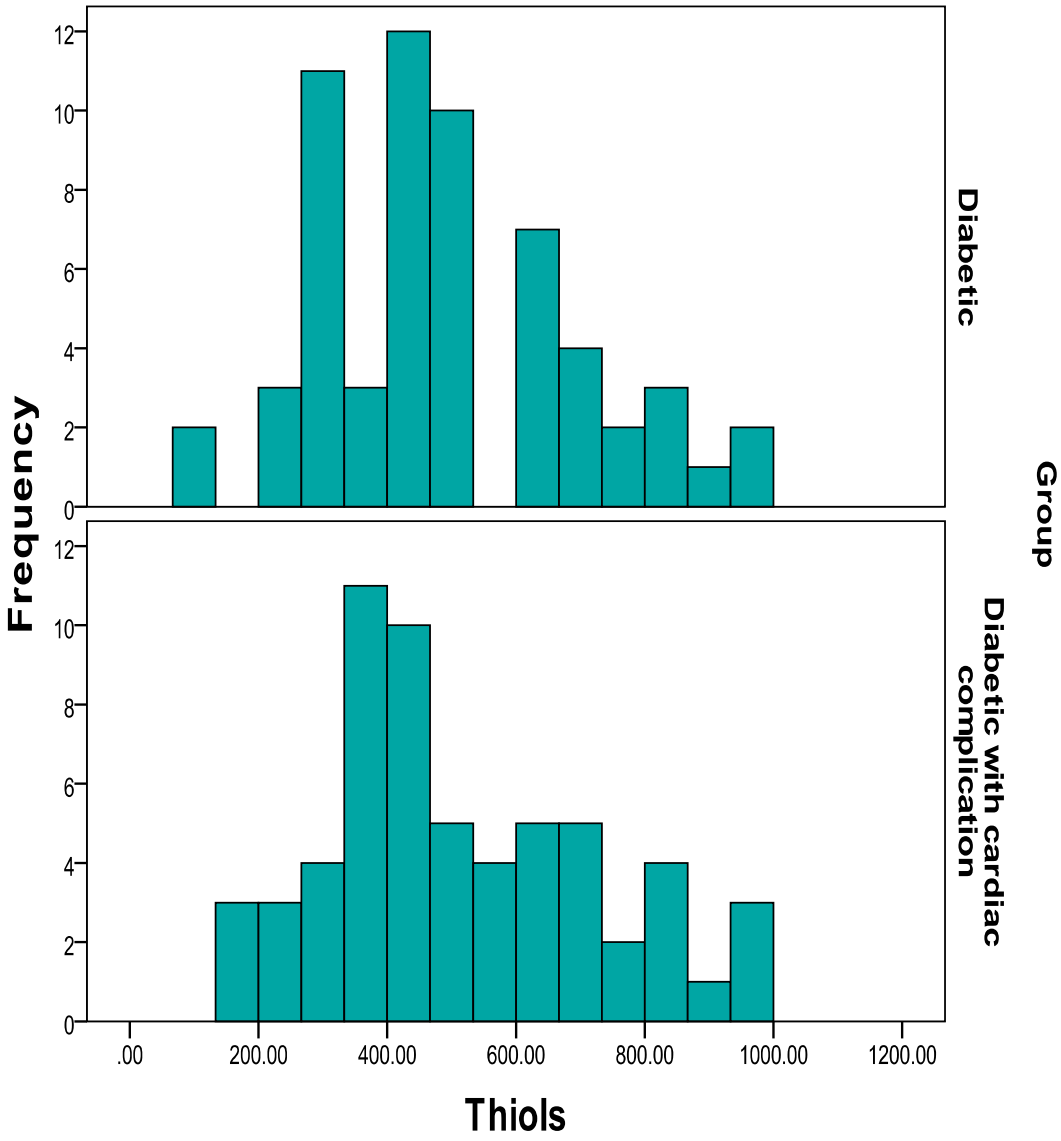


TABLE 2: Comparison of duration of diabetes mellitus, HbA1c, hsCRP, plasma total thiols between two groups

Group		Mean	Std. Deviation	Significance(2 tailed)
Duration	1	6.58	6.304	
	2	7.23	6.390	0.576
HbA1c	1	7.6935	1.93539	
	2	8.3778	1.79847	0.047
hsCRP	1	.2885	.26758	
	2	3.1970	5.83335	0.000187
Thiols	1	493.7917	204.10390	
	2	517.8683	214.74843	0.530

Group 1: Type 2 diabetic patients without cardiac complications

Group 2: Type 2 diabetic patients with cardiac complications

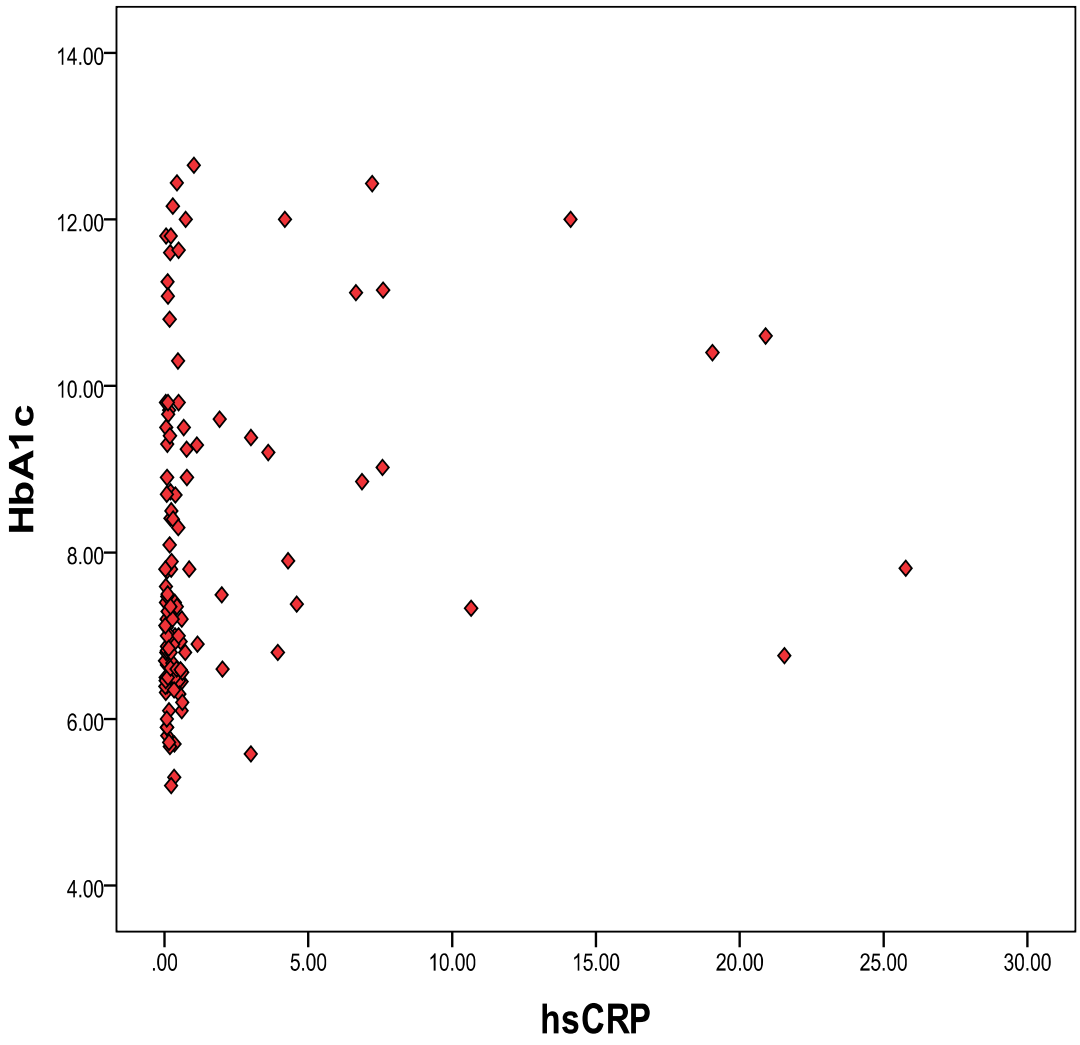
TABLE 3: Correlation of hsCRP levels with duration of diabetes, HbA1c and protein thiols.

hsCRP	r value	p value
Duration	-0.009	0.926
HbA1c	0.216	0.018
Thiols	0.373	< 0.001

TABLE 4: Correlation of protein thiol levels with duration of diabetes, HbA1c and hsCRP

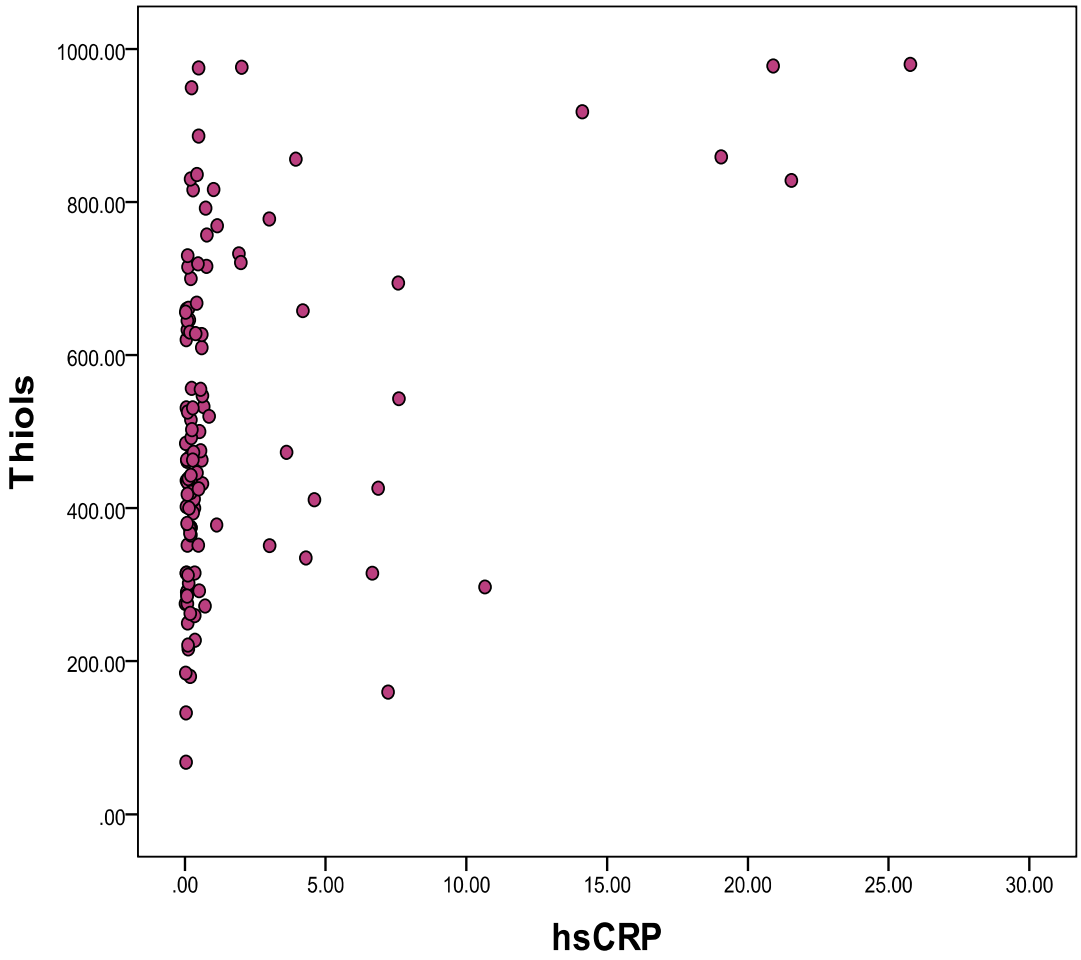
Thiols	r value	p value
Duration	-0.104	0.258
HbA1c	0.369	< 0.001
hsCRP	0.373	< 0.001

Fig 6.10: Correlation of HbA1c levels with hsCRP in study population



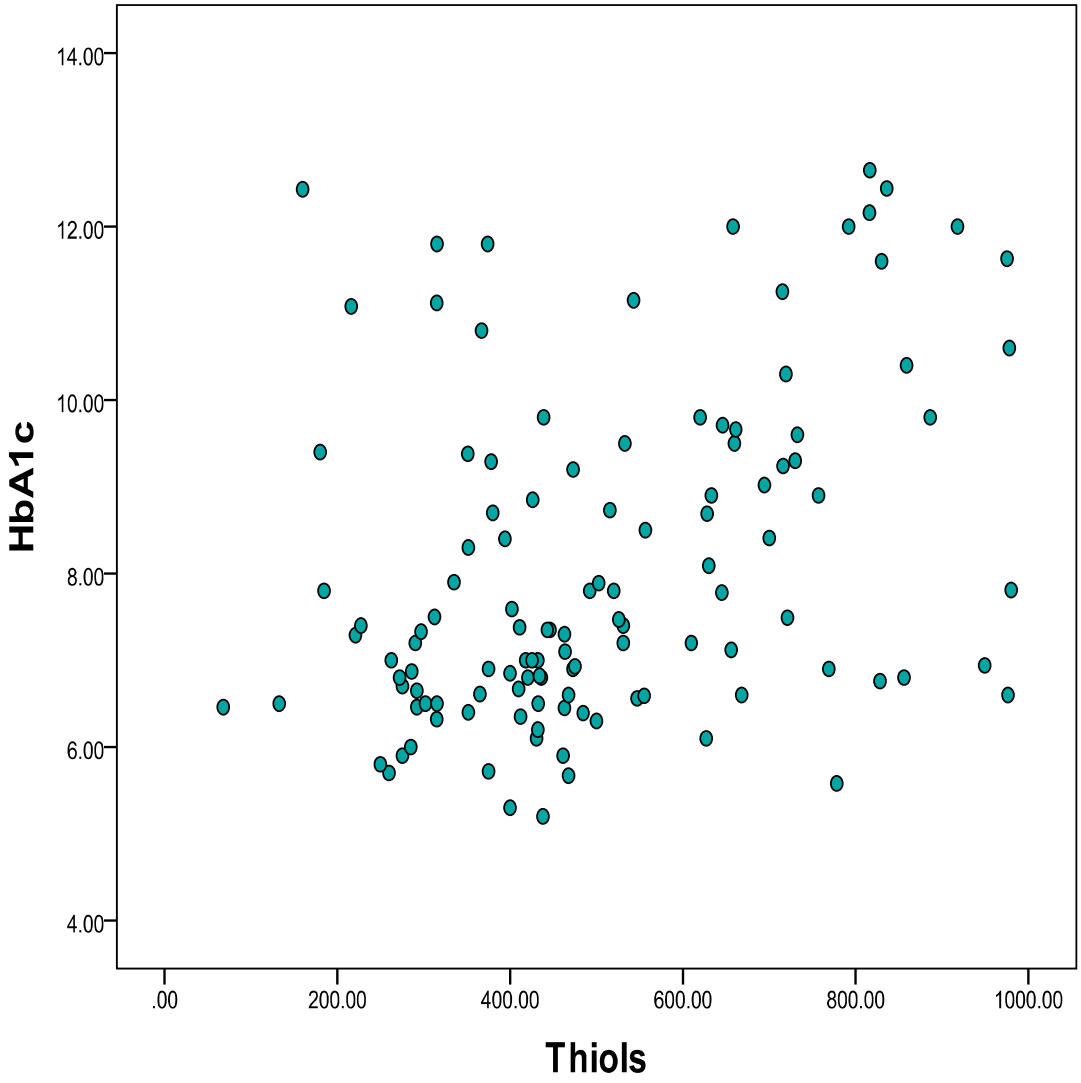
p value 0.018

Fig 6.11: Correlation of plasma total thiols with hsCRP in study population



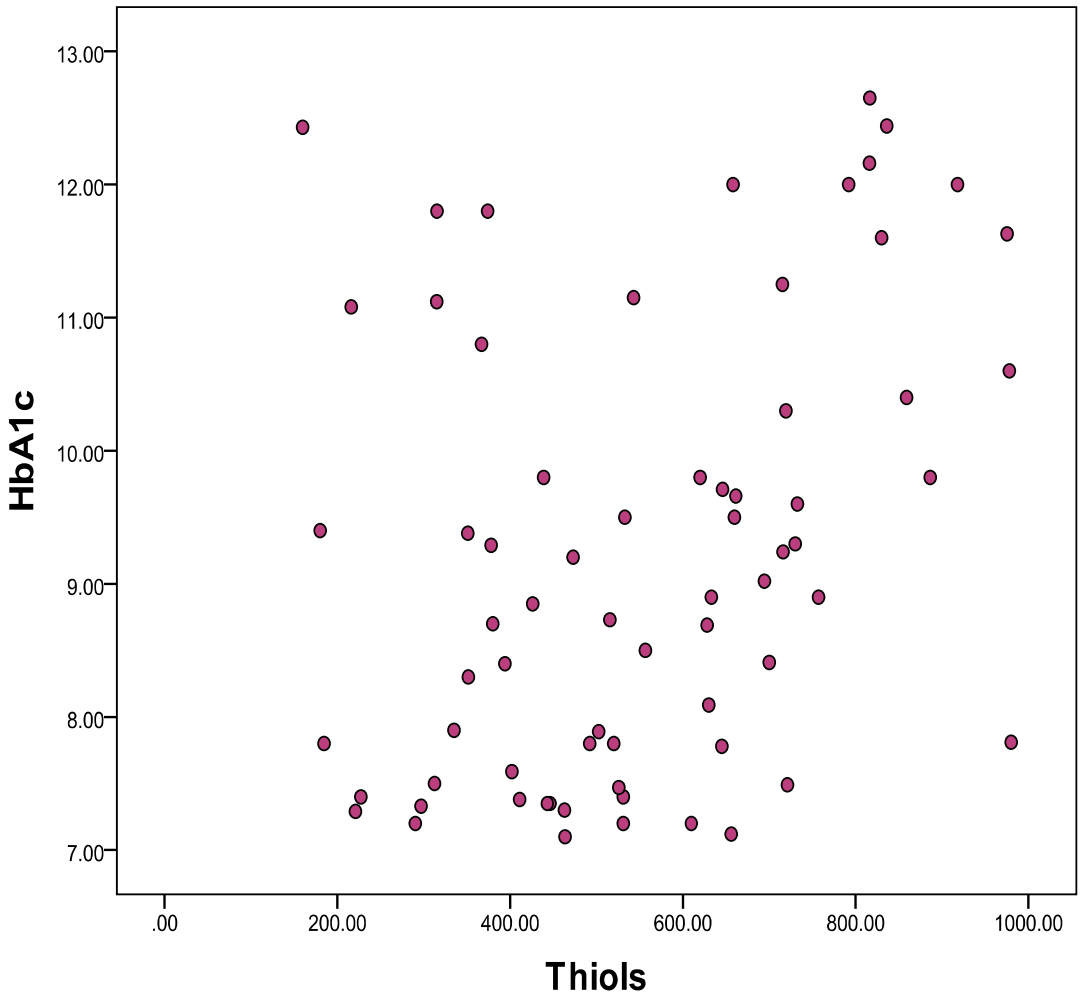
p value < 0.001

Fig 6.12: Correlation of plasma total thiols with HbA1c in study population



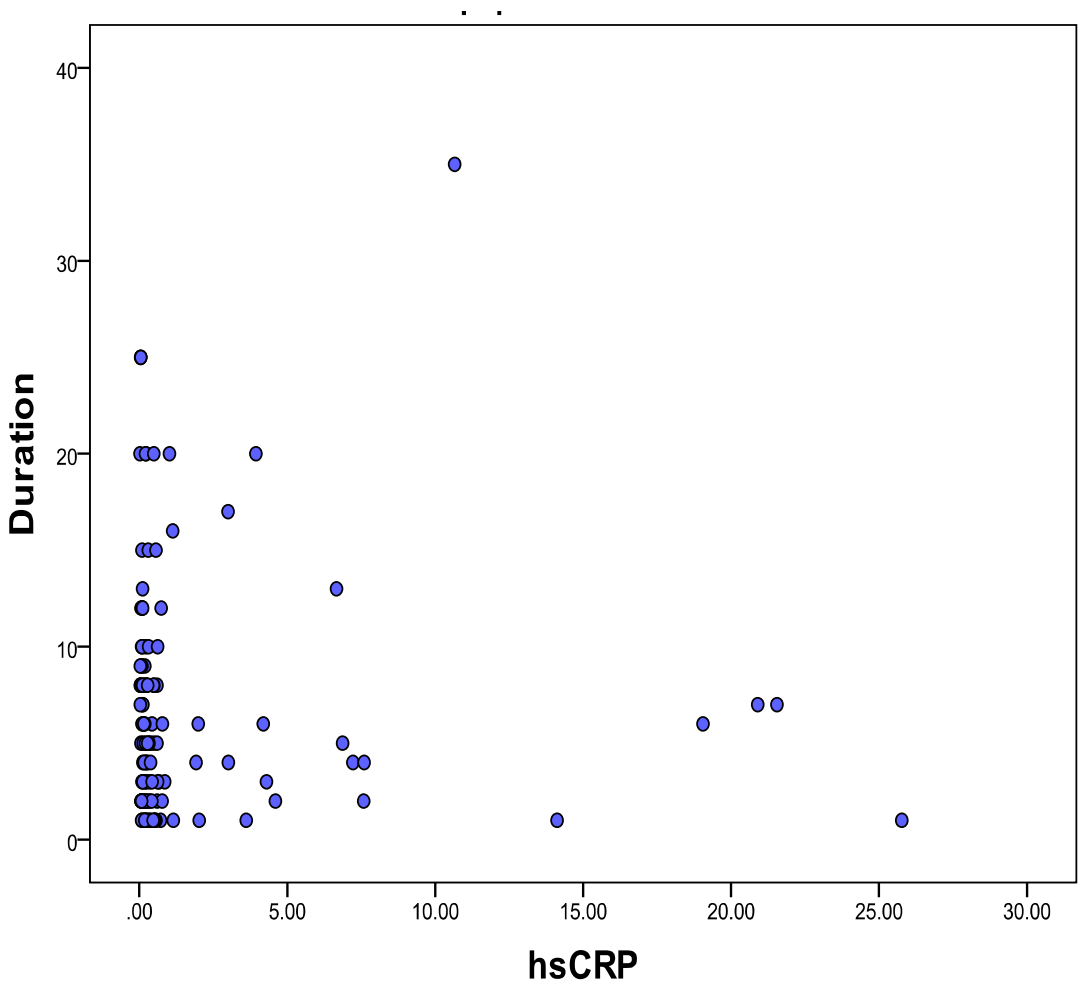
p value < 0.001

Fig 6.13: Correlation of plasma total thiols with diabetic patients with poor glycemic control (HbA1c >7%) in study population



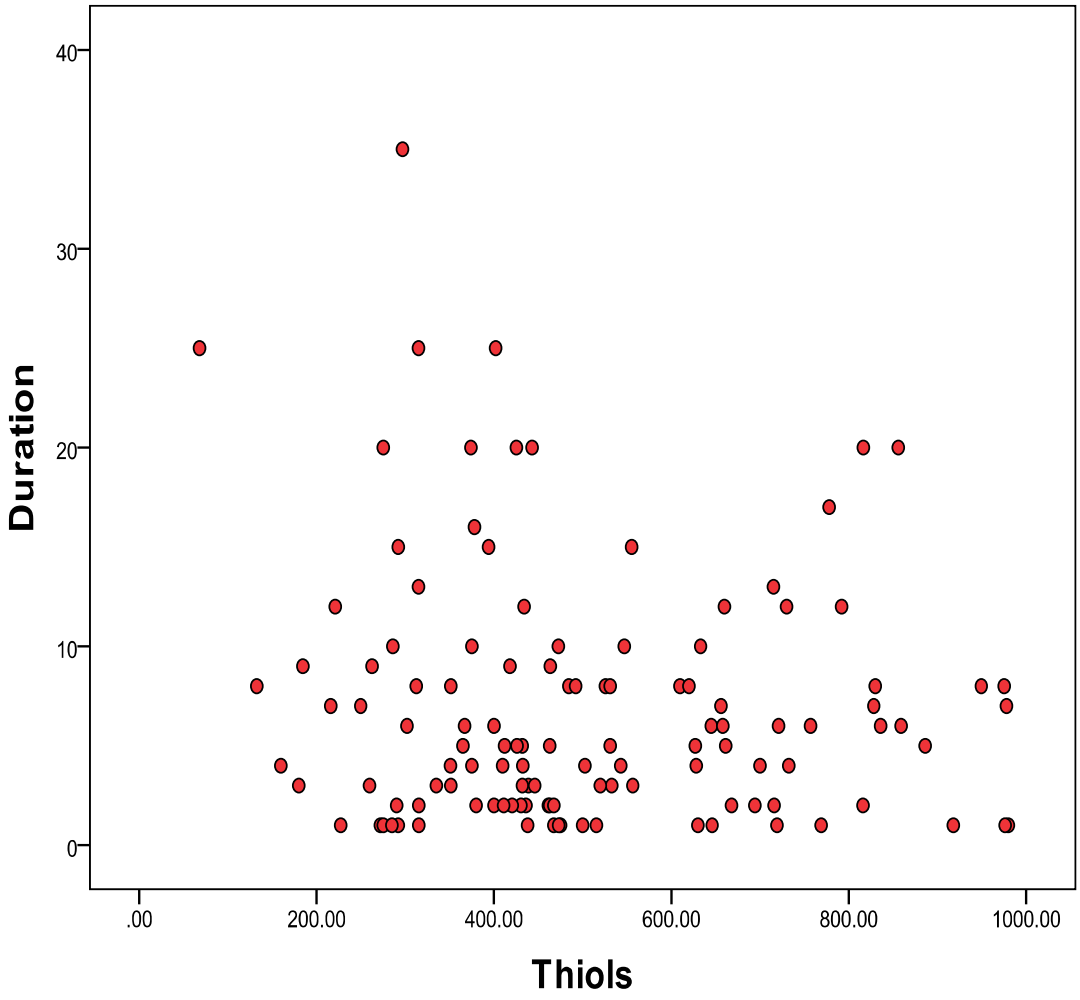
p value 0.005

Fig 6.14: Correlation of hsCRP levels with duration of diabetes mellitus in study population



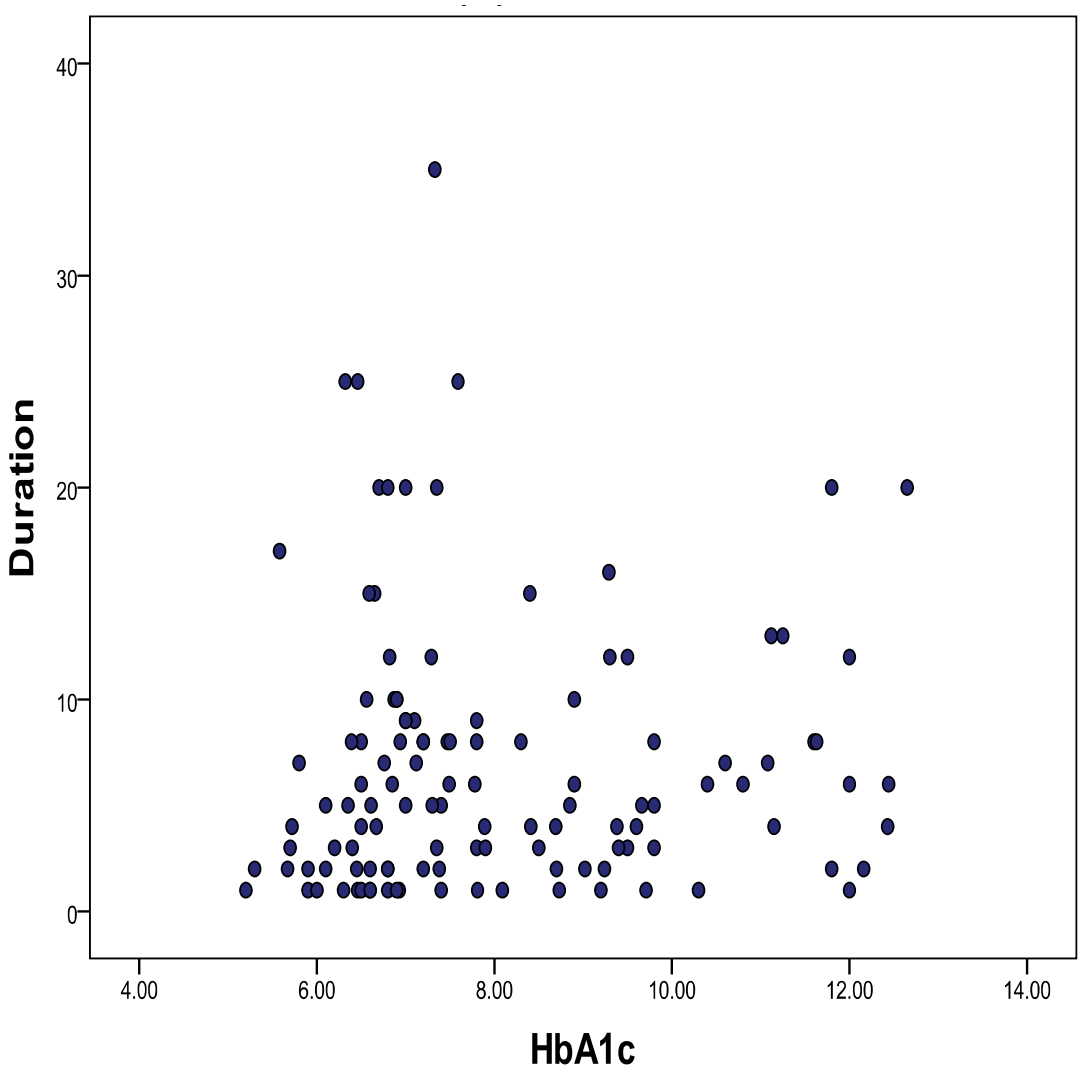
p value 0.926

Fig 6.15: Correlation of plasma total thiol levels with duration of diabetes mellitus in study population



p value 0.258

Fig 6.16: Correlation of HbA1c levels with duration of diabetes mellitus in study population



p value 0.73

7. Discussion:

Type 2 diabetes mellitus is a chronic metabolic disease and is associated with numerous micro-vascular and macro-vascular complications. This reduces the life expectancy of the patients by ten years. Various studies have shown the relationship between oxidative stress, inflammation and the pathogenesis of type 2 diabetes mellitus, implicating their role in the occurrence of complications[85, 86]. Cardiovascular complications have a major role in increasing the morbidity and mortality in type 2 diabetes mellitus patients[87]. Persistent hyperglycemic state is known to induce Oxidative stress which in turn has been shown to cause endothelial dysfunction .This initiates the events leading to the pathogenesis of micro and macro-vascular diseases. Oxidative stress is known to increase the expression of pro-inflammatory markers and pro-coagulant factors. It also induces apoptosis and impairs the release of nitric oxide[88].

This study was done to assess the oxidant and inflammatory status in type 2 diabetes mellitus patients with and without cardiac complications, by measuring their plasma protein thiols and hsCRP levels. I have also tried to find out if there is a correlation existing between these two biochemical parameters.

Fig 6.1 shows that type 2 diabetic patients selected with cardiac complications were more in 60 to 70 years of age. So independent t test was done to analyze and account for age as a confounding factor and negate it if found to be significantly interfering with the study. But on statistical analysis, the p value was found to be 0.116 which was not significant.

Sex distribution of the number of patients was found to be almost equal among the two groups. Chi square test was done for the same and the p value was found to be 0.715 which was also not significant.

Type 2 diabetic patients should undergo screening for diabetic complications as soon as they are diagnosed and yearly thereafter. The duration of diabetes mellitus from the time of diagnosis has a great influence on the development of complications.

Previous studies have shown that the development and prevalence of complications positively correlate with the duration of disease, irrespective of the patient`s age [89, 90, 91]. In this current study most of the diabetic patients without complications had been suffering from the disease for less than ten years. Diabetic patients with cardiac complications have had a longer duration of diabetes mellitus and the p value between the two groups was found to be 0.576 which is not statistically significant.

ADA has approved HbA1c ≥ 6.5% as the criteria for diagnosing diabetes mellitus. According to UKPDS study, diabetic complications can be prevented with good glycemic control[92].Stratton IM et al had shown that degree of hyperglycemia measured by plasma glucose levels or glycated haemoglobin (HbA1c) was significantly associated with the incidence of cardiovascular complications. They have concluded that 14% reduction in myocardial infarction and 37% reduction in microvascular complications in diabetic patients would be possible, with 1% reduction in average HbA1c levels[93].

In this study, most of the HbA1c values of type 2 diabetic patients without complications were between 6-8 %. Findings in this study also correlate with other studies. Independent t test was done and the p value of HbA1c levels between the two groups found to be 0.047 which was statistically significant.

C - reactive protein though an acute phase protein when measured through high sensitive assays, is fairly specific for the prediction of cardiovascular diseases. The role of hsCRP in atherosclerosis has already been studied. hsCRP being a part of innate immune response, activates complement pathway and stimulates the expression of adhesion molecules. It also recruits monocytes and enhances atherosclerosis. Rise in CRP level is also associated with endothelial dysfunction. According

to Sanchez et al, in type 2 diabetic patients with acute coronary syndrome hsCRP can be used as a marker for predicting cardiovascular death[57].

American Heart association has set hsCRP cut points to be less than 1 mg/L, 1 - 3 mg/L, and greater than 3mg/L which correspond to low-risk, medium-risk, and high risk group's respectively[56]. Out of the 60 diabetic patients without cardiac complications, 58 patients fall under low risk and 2 patients fall under medium risk groups.

Mohan et al in Chennai Urban Rural Epidemiology Study (CURES), have found that diabetic subjects with Coronary artery disease (CAD) had higher CRP levels when compared to diabetic subjects without CAD and non diabetic subjects[94]. Baig et al in a study has got a similar finding that patients with diabetic complications had higher hsCRP levels compared to those without complications and these values are far higher than the non diabetic subjects ($p<0.001$)[95].

The results of this current study have been supported by the above mentioned studies. In type 2 diabetic patients who had cardiac complications, hsCRP values were markedly elevated when compared to those without complications expressing a p value of 0.0001(highly significant).

Reactive oxygen species (ROS) can induce oxidative modification of proteins directly or indirectly by the by-products of oxidative stress. As a result protein carbonyl derivatives are formed which are considered to be the most used biomarkers for oxidative tissue damage. Early formation, greater strength and longer life span is the reason for protein oxidation products being increasingly used as markers instead of lipid peroxidation products in the demonstration of oxidative imbalance. The estimation of plasma total thiol will reveal excess free radical generation during oxidative stress. Protein thiols scavenge oxidants that initiate peroxidation. The thiol (–SH) moiety of cysteine is very much susceptible to oxidative attack and this results in the formation of disulfide bonds and thiyl radicals. Rise in oxidative protein damage and reduced antioxidative defense mechanisms are thought to be the primary pathology behind the development of diabetic complications [96].

Patients with CAD are known to have higher malondialdehyde (MDA) and lower total plasma protein thiol levels than the controls; MDA and total thiols represent the oxidative damage products of lipids and proteins respectively[97]. Jaiprakash et al have studied the plasma thiol levels in three groups namely control, diabetic patients per se and diabetic people with end stage renal disease (ESRD). The results showed that plasma thiol concentration was significantly decreased (p value < 0.005)

in diabetic patients without ESRD when compared with controls [98].There was also a significant rise in plasma thiols in diabetic patients with end stage renal disease when compared to controls which concurred with other studies[99, 100].

Rama Srivatsan et al also have studied protein thiol levels in three groups namely controls, diabetic and diabetic people with microvascular complications. According to that study there was no significant difference in protein thiol concentration between control and type 2 diabetics and also with type 2 DM with complications (P=0.79,P=0.55)[101]. Since cardiovascular complications contribute much to the mortality of type 2 diabetic patients we estimated the total plasma thiols in patients with type 2 diabetes mellitus with and without cardiac complications and found that there was no statistical significance (p value, 0.530)between the two groups.

The Insulin Resistance Atherosclerosis Study concluded that insulin resistance is related to CRP levels, fibrinogen and plasminogen activator inhibitor-1 (PAI-1). Rise in PAI-1 and CRP levels are predictors of the development of type2 diabetes[102].

Another study has correlated hsCRP with HbA1c levels in overweight type 2 diabetic female patients[103]. Bahceci M et al analysed high sensitivity CRP levels in Type 2 diabetic men without coronary heart disease , non-diabetic CHD patients and T2DM patients with CHD. He also concluded that there is a positive correlation between serum hs-CRP and HbA1c levels[104]. Finding of this current study also substantiates the results of previous studies. Pearson correlation shows a positive correlation between glycated haemoglobin (HbA1c) levels and hsCRP with r value 0.216.p value is found to be 0.018 which is statistically significant.

Previous studies have shown that measurement of intracellular and extracellular aminothiol compounds will describe the effect of oxidative stress in the body[105]. According to a study by Salman Ashfaq measurement of thiols which determines the oxidative stress predicts early atherosclerosis, which is measured by carotid intima- media thickness[106]. The current study and the analysis of the results suggests a very significant positive correlation between the hsCRP levels in plasma and the plasma total protein thiols in all the subjects who have diabetes with or without complications of diabetes with p value of < 0.001. This is suggestive of the fact that inflammatory status and oxidative stress are

proven concepts behind pathogenesis of diabetes, a chronic disease of mankind.

hsCRP is an established marker of inflammation and infections while change in thiol levels are noted in many studies involving stress induced due to oxidative changes because of persistent hyperglycemia.

One study has shown that poor glycemic control due to persistent hyperglycemia causes glycation of proteins leading to increased oxidative damage of proteins and this increases the rate of development of diabetes related complications[107].

The current study and the analysis of the results also suggests a very significant positive correlation between the blood levels of HbA1c and the plasma total protein thiols in all the subjects who have diabetes with or without complications of diabetes with p value of < 0.001. Further analysis of thiol levels with glycemic status shows that poor glycemic control with HbA1c > 7 % shows a significant positive correlation with thiol levels having a p value of 0.005 than with that of good glycemic control.

HbA1c level in the blood is not only a direct predictor of the risk of complications in diabetes, but also a prognostic marker and treatment

guide for Diabetes and hence the results are suggestive of the fact that as HbA1c levels rise, the severity of complications also rise which indirectly suggests the inflammatory status /oxidative stress state is prevalent in the patients under study.

Change in thiol levels are noted in many studies due to oxdative stress resulting from persistent hyperglycemia. In this current study the change in thiols is in the upward direction which facilitates the idea of induction in the synthesis of thiols to counter the oxidants. Some studies on the other hand have shown a fall in thiol levels due to oxidative damage which has been explained by consumption and near exhaustion of thiols in the process of neutralizing the oxidant radicals[108].

8. Conclusion:

Diabetes mellitus is a group of metabolic disease presenting with symptoms of hyperglycemia which may be either due to defective secretion of insulin by pancreas or resistance to insulin action or both. The current study tried to estimate plasma total thiols, hsCRP and HbA1c levels in type 2 diabetic patients without complications and type 2 diabetic patients with cardiac complications. This study also tried to find out if there is a correlation existing between each other in the study groups.

- The results suggest that there is a significant elevation in HbA1c and hsCRP levels in type 2 diabetic patients with cardiac complications when compared to diabetics without complications.
- No significant differences in plasma total thiols have been noted between the two groups.
- There is a very significant positive correlation between hsCRP, plasma total thiols and HbA1c levels in type 2 diabetic patients. The findings of this study suggest that poor glycemic control, oxidative stress and inflammation contribute to the pathogenesis of diabetic complications.

Limitations of the study:

- Small sample size due to financial constraints.
- Type of treatment given for type 2 diabetic patients with and without complications would differ and the treatment options within the same group may be different. The effect of drugs used is not taken into consideration. If those drugs per se had a say on the inflammatory status of the patient it would not be showing up on this current study.
- Use of statins in diabetic patients with cardiac complications may alter the inflammatory status of the patient.

9. Summary:

Diabetes mellitus is a growing non communicable disease presenting with symptoms of hyperglycemia due to reduced insulin secretion, defective insulin action or a combination of both. If not detected earlier patients may present with diabetic complications. As the duration of diabetes mellitus increases, the risk of developing complications also increases. It is associated with a number of micro-vascular and macro-vascular complications which adds to the burden of the disease. Diabetes mellitus is considered to be coronary heart disease equivalent. Cardiovascular diseases and mortality due to it are much greater in people with type 2 diabetes mellitus than that of non- diabetic individuals.

Many studies have shown the role of oxidative stress and inflammation in type 2 diabetes mellitus and its complications with the help of oxidative and inflammatory biomarker assays. Few studies have been conducted to correlate plasma total thiols between type 2 diabetes mellitus patients with and without cardiac complications.

This study was done to measure plasma total thiols, hsCRP and HbA1c levels in type 2 diabetic patients without complications and type 2 diabetic patients with cardiac complications. This study also tried to find out if there is a correlation if any, existing between them in the study

population.HbA1c, plasma total protein thiols, plasma hsCRP were estimated in 60 type2 diabetic patients without complications and 60 type2 diabetic patients with cardiac complications. Plasma total protein thiols was estimated spectrophotometrically by using Dinitrobenzene (DTNB)-Ellman's method. Plasma hsCRP by particle enhanced turbidimetric assay.HbA1c by turbidimetric inhibition immunoassay.

HbA1c (p - 0.047) and plasma hsCRP(p- 0.0001) were significantly higher in type2 diabetic patients with cardiac complications. There is also a very significant positive correlation between the blood levels of HbA1c with the plasma total protein thiols (p value < 0.001) and hsCRP (p value 0.018). In addition there is a very significant positive correlation between the plasma levels of hsCRP and the plasma total protein thiols with a p value of < 0.001.

Role of oxidative stress and inflammation are established in the pathogenesis of diabetic complications and persistent hyperglycemia contributes to these changes. This is the reason for increased HbA1c and plasma hsCRP in type 2 diabetic patients with cardiac complications. Change in thiol levels are due to the increased synthesis to compensate for the loss incurred during neutralization of the oxidants.

10. Future scope of the study:

- Many of the disadvantages can probably be negated if the sample size is substantially increased
- Plasma total thiols and hsCRP may be used as a prognostic marker for survival of the patient
- Genetic analysis for the proteins like CRP might throw some light into the pathogenesis of type 2 diabetes mellitus and its complications.

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ANNEXURE

Master chart for Type 2 Diabetic patients without complications

S.No	Age	Sex	hsCRP	Duration	HbA1C	Thiols
1	63	M	0.1	7	5.8	249.75
2	42	M	0.16	1	9.71	646
3	50	F	0.6	8	7.2	609.75
4	40	F	0.72	1	6.8	272
5	35	M	0.06	2	11.8	315.25
6	35	M	0.26	1	6.6	467.5
7	55	M	0.21	4	8.41	700
8	75	M	0.09	15	6.65	292
9	39	M	0.29	2	12.16	816
10	59	M	0.04	8	6.5	132.5
11	69	F	0.02	20	6.7	275.25
12	65	M	0.05	25	6.32	315
13	37	F	0.08	2	5.9	461.25
14	55	M	0.21	10	6.9	375
15	35	F	0.6	2	6.45	463
16	64	M	0.37	5	7	432
17	63	F	0.09	3	6.4	351.5
18	60	M	0.2	8	11.6	830
19	36	F	0.34	2	5.3	400
20	56	F	0.36	1	7.4	227.25
21	65	F	0.07	2	7.2	290.25
22	49	F	0.35	3	5.7	259.75
23	45	M	0.23	1	5.2	438
24	36	M	0.24	8	6.94	949.5
25	65	F	0.08	10	6.87	286
26	48	F	0.49	5	9.8	886.25
27	68	M	0.05	5	7.4	531
28	52	M	0.03	8	6.39	484.5
29	56	M	0.14	6	6.5	302
30	59	M	0.56	1	6.93	475

S.No	Age	Sex	hsCRP	Duration	HbA1C	Thiols
31	39	M	0.67	3	9.5	532.75
32	72	F	0.05	8	9.8	620
33	57	F	1.02	20	12.65	816.5
34	40	F	0.06	2	6.8	436
35	38	M	0.16	2	6.1	430.5
36	38	M	0.51	1	6.46	292
37	60	M	0.09	10	8.9	633
38	74	M	0.06	12	9.5	659.75
39	49	F	0.28	4	6.67	409.75
40	51	F	0.11	13	11.25	715.25
41	67	F	0.04	25	6.46	68
42	44	F	0.35	1	6.5	315.25
43	35	F	0.19	2	5.67	467.5
44	58	M	0.52	1	6.3	500
45	47	M	0.74	12	12	792
46	59	M	0.77	2	9.24	716
47	44	M	0.12	4	6.5	432.5
48	58	F	0.09	1	5.9	275.25
49	56	M	0.21	1	8.73	515.5
50	54	F	0.13	5	9.66	661.25
51	49	F	0.49	8	11.63	975.25
52	69	M	0.05	25	7.59	402
53	40	F	1.15	1	6.9	769
54	58	F	0.09	12	6.82	434
55	63	M	0.07	9	7.1	463.5
56	42	F	0.6	5	6.1	627
57	63	F	0.31	10	6.9	472.75
58	47	F	0.33	5	6.35	412
59	64	M	0.86	3	7.8	520
60	43	F	0.1	12	9.3	730

Master chart for Type 2 Diabetic patients with Cardiac complications

S.No	Age	Sex	hsCRP	Duration	HbA1C	Thiols
1	55	F	0.09	6	7.78	645
2	64	M	1.13	16	9.29	378
3	60	F	0.08	1	6	285
4	53	M	0.19	3	9.4	180
5	59	M	0.1	8	7.47	525.75
6	60	F	0.12	7	11.08	216
7	58	F	0.22	20	11.8	374
8	67	M	0.22	2	6.8	420.5
9	60	F	0.21	5	6.61	365
10	48	M	0.23	8	7.8	492.25
11	64	M	0.19	9	7	262.5
12	45	F	19.05	6	10.4	859
13	43	M	6.87	5	8.85	426
14	65	M	20.9	7	10.6	978
15	65	F	0.43	6	12.44	836
16	46	M	0.09	9	7	418
17	50	F	0.24	3	8.5	556.5
18	56	M	0.25	4	7.89	502.6
19	57	F	0.11	8	7.5	312.5
20	63	M	0.42	2	6.6	668
21	40	F	3.61	1	9.2	473
22	55	M	7.22	4	12.43	159.75
23	42	M	0.12	3	9.8	438.75
24	64	F	0.78	6	8.9	757
25	50	M	6.66	13	11.12	315
26	50	F	1.92	4	9.6	732.5
27	50	M	4.6	2	7.38	411
28	68	M	0.3	15	8.4	394
29	58	F	0.16	4	5.72	375
30	60	M	0.28	5	7.3	463

S.No	Age	Sex	hsCRP	Duration	HbA1C	Thiols
31	55	F	0.62	3	6.2	432
32	35	F	14.12	1	12	918
33	65	M	0.48	8	8.3	351.5
34	46	F	0.18	6	10.8	367
35	65	M	0.62	10	6.56	547
36	37	M	4.19	6	12	658
37	43	M	0.03	7	7.12	656
38	49	M	7.58	2	9.02	694.25
39	64	F	4.3	3	7.9	335
40	46	M	0.18	1	8.09	630
41	60	M	1.99	6	7.49	721
42	51	F	3.01	4	9.38	351
43	64	F	0.15	6	6.85	400
44	67	M	0.11	12	7.29	221
45	55	F	0.38	4	8.69	628
46	68	F	21.55	7	6.76	828.25
47	52	F	3.94	20	6.8	856
48	60	F	0.28	8	7.2	531
49	35	F	7.6	4	11.15	543
50	61	M	0.43	3	7.35	446
51	65	F	0.21	20	7.35	443
52	43	F	0.03	9	7.8	184.5
53	72	M	3	17	5.58	778
54	48	F	0.47	1	10.3	719.25
55	46	M	0.08	2	8.7	380
56	74	M	25.77	1	7.81	980
57	66	M	0.56	15	6.59	555.25
58	69	M	10.66	35	7.33	297
59	71	F	0.49	20	7	425.25
60	35	F	2.02	1	6.6	976.25

DATA COLLECTION TOOL (QUESTIONNAIRE)

1. Patient's name :
2. Patient's ID :
3. Age :
4. Sex :
5. History of illness :

5.1. Diabetes : Type 1 Type 2

5.2. Duration of diabetes :

5.3. Hypertension : 1. Yes 2. No

5.4. Smoking history :

If yes, pack years :

5.5. H/O myocardial infarction/Angina/LV dysfunction:

1. Yes 2. No

5.6. Vision :

5.7. Motor/sensory functions :

5.8. History of any other illness : 1. Yes 2. No

If yes, Details :

5.9. Details of treatment :

5.10. Lab values :