Benghazi University

Faculty of Medicine

Role of Glutathione-S-Transferase P1 genetic variants in the etiopathogeneses of type 2 Diabetes Mellitus and its effect on the glycemic control parameters

" A dissertation submitted in partial fulfillment for the requirement for the degree of master of science in biochemistry to the faculty of medicine, Benghazi university, Benghazi, Libya"

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2016

DEDICATION

TO MY PARENTS

AND MY FAMILY

WITH LOVE

ACKNOWLEDGMENENT

In the beginning, I would like to present my thankfulness and greatest glory to Allah who gave me power to complete my thesis and who always behind all successful achievement I got in my life.

I am honored to remember the people whom without their help the completion of my thesis would not have been possible, while at the same time ask forgiveness of anybody that was not acknowledged for providing assistance to me no matter how small.

I would like to express my deepest gratitude to my supervisor **Professor Abdalla Al-Jarari** for his guidance, caring, patience and providing me with an excellent atmosphere for doing research.

My deepest gratitude to my supervisor **Professor Farag El-Shaari** for his guidance and encouragements and continuous support throughout the project to complete the study.

A deep and grateful thanks to **Mr. Wael El-Hawari,** for his encouragments and sincere help, and providing me with laboratory facilities to complete my experiments.

A deep and grateful thanks to **Dr. Ragab Roaeid,** and laboratory staff of Benghazi Center for Diagnosis and Treatment of Diabetes for their sincere help**.**

A deep and grateful thanks to **Dr. Abdalla Bashein** for his sincere help.

A deep and grateful thanks to **Mr. Omer Belhassan** for their sincere help.

I would like to thank to all staff in the department of Biochemistry for their encouragement and support.

I am grateful to Biotechnology Center (Benghazi Branch) for providing me with laboratory facilities to carry out the experiments.

I am grateful to Benghazi Center of Infectious Diseases and Immunology for providing me with laboratories facilities to carry out the experiments.

Last but not the least; I am deeply grateful to my family and all my friends who support me during the period of my study and beyond.

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ABBREVIATIONS	
DM	Diabetes Mellitus
WHO	World health organization
NDDG	National diabetes Data Group
OGTT	Oral glucose tolerance test
ADA	American diabetes association
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-Insulin dependent diabetes mellitus
NADH	Reduced form of Nicotinamide adenine dinucleotide
FADH2	Reduced form of flavin adenine dinucleotide
IRS	Insulin receptor substrate
HbA1c	Glycosylated hemoglobin
SH2	Scr homology 2 domain
PIP3	Phosphatidylinositol(3,4,5) triphosphate
Ser/Thr kinase	Serine/threonine kinase
PDK1	Phosphatidylinositol dependent protein kinase
AKT	Protein kinase B

GSK3B	Glycogen synthase kinase 3b
AS160	AKt substrate of 160kDa
PDX-1	Pancreatic and duodenal homeobox-1
Bcl2	B-cell lymphoma2
ROS	Reactive Oxygen Species
AGE	Advanced glycosylation end products
TNFα	Tumor Necrosis Factor α
MODY	Maturity onset diabetes of young
GLUT	Glucose transporter
GST	Glutathione-s-transferase
3L3-Li adipocytes	A Cell line derived from(mouse)3L3 cells
HDL	High density lipoprotein
VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
BMI	Body mass index
BP	Blood pressure
FBG	Fast blood glucose
SD	Stranded deviation

Ob/Ob mice	Leptin-deficient mouse

Abstract

Introduction: Diabetes Mellitus is a group of metabolic diseases characterized by chronic hyperglycemia. Type 2 diabetes is the most common form and comprises of 90% of people with diabetes around the world, it characterized by insulin resistance or relative insulin deficiency.

Glutathione-S-transferases (GSTs) are key enzymes of phase II related to the metabolism of numerous genotoxic compounds. Glutathione-S-transferases M1 (GSTM1) and T1 (GSTT1), the important subtypes of GSTs enzymes, are reportedly involved in detoxification of reactive oxygen species, which are considered to play a key role in the occurrence of various endocrine-related cancers. Another member of the glutathione-S-transferase (GST) family, GSTP1, which is located at 11q13, has a role in the detoxification of electrophilic compounds by glutathione conjugation . It has been found that two genetic polymorphisms in exon 5 and exon 6, lead to amino acid substitutions. However, only the transition in exon 5 was linked to activity of enzymes since this is located within the region coding for the enzyme's active site. The genetic change in exon 5 at the site -313, results in polymorphism at codon 105, where an adenosine-to-guanidine ($A > G$) transition causes an Ile-to-Val substitution.

Several investigators have determined the clinical or genetic factors associated with T2DM with interests to detoxification agents.

Patients and methods: In the present study, a total of 67 type2 Libyan diabetes patients were recruited from the Benghazi center for diagnosis and treatment of diabetes, 26 apparently healthy age and sex-matched individuals were selected from the high institute of medical professions' Benghazi to serve as controls. Clinical information and medical history were obtained through patients' interview.

Venous blood samples were drawn from all the participants after at least 10 hours fasting, and analyzed for blood glucose, HbA1c, total cholesterol, triacylglycerol, and HDL-c using an auto analyzer with enzymatic techniques. LDL cholesterol was calculated according to Friedewald equation.

The data were analyzed using SPSS version 18, *P* values < 0.05 were considered to be statistically significant.

DNA is extracted from whole blood samples by using QIAamp DNA Blood Mini Kit(250), then amplified the desired gene with the specific primers using PCR thermal cycler TC 5000 and digested the PCR products with restriction enzyme ALW26I overnight. To detect the GSTP1Ile105Val genotype loading digested products on 2.5%agarose gel electrophoresis.

Results: In the present study, diabetic patients had significantly higher fasting blood glucose, HbA1c, total cholesterol, triacylglycerol, and LDL-c, and lower HDL-c than control patients group.

Healthy controls were carrying more G allele(val) than the type 2 diabetes mellitus as illustrated after running of digested PCR products on agarose gel (91bp+85bp) and in analysis by high resolution melting(HRM-RTPCR).

Conclusion: In the present study, GSTP1Ile105Val may not play role in the etiopathogeneses of diabetes mellitus and on glycemic control parameters.

1-Review of literature

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is probably one of the oldest diseases known to people(Olokoba et al., 2012). World Health Organization defines Diabetes mellitus as a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood ,which in turn lead to chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism(Joshi and Shrestha, 2010, Kumar and Clark, 2002, Gavin III et al., 1997, Beverley and Eschwège, 2003, Lindberg et al., 2004). As the disease progresses tissue or vascular damage ensues leading to severe diabetic complications such as retinopathy (Bearse et al., 2004, Hove et al., 2004), neuropathy (Seki et al., 2004, Moran et al., 2004), nephropathy (Huang et al., 2002, Looker et al., 2003), cardiovascular complications (Svensson et al., 2004, Saely et al., 2004) and ulceration (Wallace et al., 2002, Control and Prevention, 2003).

Type1 and type2 are the most known types of diabetes. genetic susceptibility, auto-immunity, some viruses and intra-uterine environment have been attributed to diabetes type1 (Epstein et al., 1994, Nakayama et al., 2005, Bach, 2002). Whereas, type2 is considered life-style mediated and associated with obesity, high serum of low density lipoprotein, though role of genetic susceptibility and intra-uterine development can also be denied(Bodansky et al., 1992, Fisher and Fvier, 2002).

1.1.1 Epidemiology

It is estimated that 335 million people had DM in 2011; by 2020 this would have risen to 521 million. The number of people with type 2 DM is increasing in every country with most of people with DM living in low-and middle-income countries. DM caused 4.6million deaths in 2011(Chamnan et al., 2011).It is estimated that 438 million people would have type2 DM by the year 2030 (Zimmet et al., 2001). The incidence of type 2DM varies substantially from one region to the other as a result of environmental and lifestyle risk factors (Zimmet et al.,2001). In a study of 952 type 2 diabetic patients in

Benghazi, about 68.7% had long term complications of diabetes. 14.9% of patients had coronary heart disease, 30.6% had diabetic retinopathy, 47.1% had peripheral neuropathy, 25.8% had macroalbuminuria, 15.2% had peripheral arterial disease, and 13.1% had cataract (Kadiki and Roaeid, 2001). Literature search has shown that there are few data available on the prevalence of type2 DM in Africa as a whole. Studies examining data trends within Africa point to evidence of an increase in prevalence in both rural and urban setting, and affecting both gender equally (Mbanya, 2009).

The majority of the DM burden in Africa appears to be type2 DM, with less than 10% DM cases being type1 DM.

1.1.2 Diagnosis and classification:

The diagnostic criteria and the classification of diabetes was first put forward by the World health organization (WHO) in 1965 (Organization, 1965) then by the National Diabetes Data Group (NDDG) in 1979 (Group, 1979), and this was followed by simplified recommendation by the WHO in 1980. These WHO recommendations were modified slightly in 1985 (Vftter, 1986). The latest recommendations have been published by the American Diabetes Association (ADA) in 1977 and by the WHO in 1999. Both groups agree on the recommendations and criteria (Gavin III et al., 1997, Assal and Groop, 1999).

According to the ADA recommendation changes in 1997, the fasting glucose concentration should be used in routine screening for diabetes as well as epidemiological studies; the threshold for fasting glucose was changed from 140mg/dl to 126mg/dl; however the 2-h glucose criterion remains as 200mg/dl. For the diagnosis of diabetes, at least one criteria must apply:

- Symptoms of diabetes (polyuria, polydipsia, unexplained weight loss, etc) as well as causal plasma glucose concentration= 200mg/dl.
- Fasting plasma glucose= 126mg/dl, with no caloric intake for at least 8h.

2-h plasma glucose= 200mg/dl during an oral glucose tolerance test (OGTT), with the glucose load containing 75g anhydrous glucose in water.

The WHO diagnosis and classification of diabetes mellitus (1999) are identical to those of ADA, a fasting glucose=126mg/dl and/or a 2-h glucose=200mg/dl. The report states that diagnosis should not be based on a single glucose determination but

requires confirmatory symptoms or blood/plasma determination. Ideally, therefore, both the 2-h and fasting value should be used.

Diabetes mellitus may be categorized into several types but the two major types are type1and type2(Zimmet et al., 2004). On the basis of aetiology, the term type1 and type2 were widely used to describe IDDM and NIDDM, respectively; other specific types of diabetes gestational diabetes are given in Table1. The term juvenile-onset diabetes has sometimes been used for IDDM and maturity-onset for NIDDM.

Table(1.1)classification of Diabetes

Type1(1a,1b)	β-cell destruction with little or no endogenous insulin secretory capacity. Autoimmune, Idiopathic
Type2	Ranges from relative insulin deficiency to disorders of insulin secretion and insulin resistance.
Other specific types	Genetic defects of β-cell function. Genetic defects in insulin secretion. Diseases of the exocrine pancreas, Endocrinopathies, Drug-induced or chemical induced infections(congenital rubella, cytomegalovirus and others). Uncommon forms of immune-mediated diabetes. Other genetic syndromes sometimes associated with diabetes. gestational diabetes.

1.1.3 Symptoms:

Acute symptoms of diabetes are due to severe hyperglycemia and include polyuria, polydipsia, polyphagia, weight loss and blurred vision. Patients may exhibit impaired growth and increased susceptibility to infections. Acute marked hyperglycemia may lead to diabetic ketoacidosis (DKA) in type 1 diabetes or to the hyperglycemic hyperosmolar nonketotic syndrome (HHNS) in type 2 diabetes.

Chronic symptoms of diabetes are due to vascular damage from persistent hyperglycemia. Vascular damage leads to end-organ damage. Other conditions associated with diabetes, such as hypertension, dyslipidemia (as well as smoking) accelerate the development of vascular damage and the chronic complications of diabetes, which are the following

1.1.3.1 Microvascular

Microvascular complications are a significant cause of morbidity. Persistent hyperglycemia is the major cause for the microvascular complications which include

- retinopathy with potential loss of vision
- nephropathy leading to kidney failure
- peripheral neuropathy leading to pain, foot ulcers, and limb amputation
- autonomic neuropathy causing gastrointestinal, genitourinary, cardiovascular symptoms and sexual dysfunction

1.1.3.2 Macrovascular

Macrovascular complications are the main cause of mortality. Although persistent hyperglycemia may contribute to macrovascular complications, it is the associated conditions (hypertension, dyslipidemia, smoking) that account for the burden of the macrovascular complications.

- coronary heart disease which is the major cause of death for patients with diabetes
- peripheral vascular disease

Unfortunately, many patients with diabetes remain asymptomatic for long periods, so that the first presentation of the disease is frequently a chronic complication. Indeed, about 50% of newly diagnosed type 2 diabetes will have developed a vascular symptoms.

People living with type 2 DM are more vulnerable to different forms of short-and long-term complications, which often lead to their premature death. This tendency of increased morbidity and mortality is seen in patients with type 2 DM because of the

commonness of this type of DM, its insidious onset and late recognition, especially in resource-poor developing countries like Africa(Azevedo and Alla, 2008).

1.2 The endocrine Pancreas:

The endocrine pancreas consists of the islets of langerhans, which are small endocrine glands scattered throughout the pancreas. The four different types of islets and its secretory products are shown in table (2.1)

Table(2.1) Endocrine cell type in pancreatic islets of langerhans

Islet cell type	Secretory products
A cell(alpha)	Glucagon
B cell(beta)	Insulin
D cell(delta)	Somatostatin
F cell	Pancreatic polypeptide

In the average individual, approximately 40-50 units of insulin are secreted daily into the portal circulation. 1/3-1/2 of total daily insulin is basal insulin, which is secreted in the fasting state. The rest is secreted as bolus (stimulated) insulin in response to exogenous stimuli(Pittas and Greenberg, 2003, Genuth et al., 2003).

1.2.1 Function:

The main function of Beta-cell is to synthesize and secrete insulin in order to maintain circulating glucose levels within physiological range. Although there exist many triggers of insulin secretion like amino acids such as leucine, glutamine in combination with leucine, nonesterified fatty acids, hormones, neurotransmitters and drugs (sulfonylurea,glinides), glucose represents the main physiological insulin secretagogue (MacDonald et al., 2005).

According to the most widely accepted hypothesis, insulin secretion is a multistep process initiated with glucose transport into Beta cell through specific transporters (GLUT1 and GLUT2) and phosphorylation by glucokinase, which directs metabolic flux through glycolysis, producing pyruvate (Matschinsky, 1990). Pyruvate then enters the mitochondria and is decarboxylated to acetyl-coA, then enters the

tricarboxylic acid cycle. The sequence of the tricarboxlic acid cycle are followed by synthesis of reducing equivalents (NADH,FADH2) and transfer them to electron transport chain (MacDonald et al.,1991). These events result in an enhanced ratio of ATP to ADP ratio in the cytoplasm, which determines the closure of the ATP-sensitive K+ channels, depolarization of the plasma membrane, influx of extracellular Ca^{2+} and activation of exocytosis which ends in fusion of insulin granules to the beta-cell membrane (MacDonald et al., 1991, Seino et al., 2010).

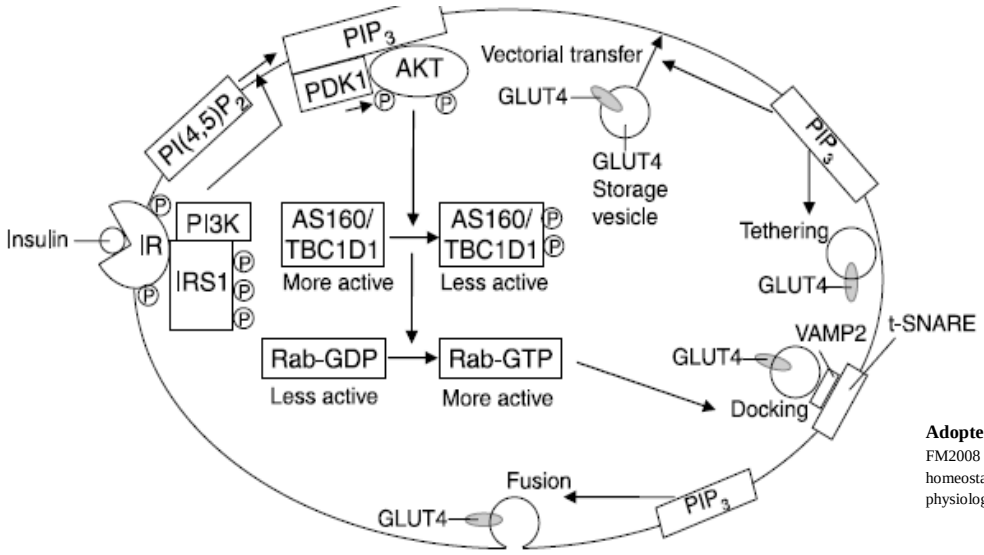
Under physiological conditions, there is a hyperbolic relation between insulin secretion and insulin sensitivity. Classically, glucose-stimulated insulin secretion is characterized by a first phase, which ends within a few minutes, decreases glucose concentration and impairment of first phase secretion is a predictor of future type1 and type2 risk and more prolonged second phase in which insulin is released proportionally to the plasma glucose (Del Prato et al., 2002). In addition, it has been demonstrated that the release of insulin is oscillatory, with relatively stable rapid pulses occurring at every 8-10 minutes (Pørksen, 2002).

1.3 Insulin signaling:

Human insulin receptor, a heterodimer, is composed of two extracellular α-subunits and two transmembrane β-subunits (White and Kahn, 1994). The α-subunit contains the extracellular ligand-binding domain that regulates intracellular tyrosine kinase activity of β-subunits (Sciacca et al., 2003, McKern et al., 2006, Ward et al., 2007). The ligand binding increases flexibility of the activation loop to allow ATP to enter the catalytic site and stabilize the activation loop in the active conformation by autophosphorylation (Hubbard et al., 1994). In higher animals, activated insulin receptor phosphorylated the tyrosine residues in cellular substrates, including insulin receptor substrate(IRS)1,IRS2,IRS3,or IRS4, or other scaffold proteins (White, 2003).

IRS proteins have tyrosine phosphorylation sites coordinate downstream signaling cascades by binding to the SH2 domains in common effector proteins. The classic insulin-like signaling cascades involve the production of PI(3,4,5)P3 by the phosphatidylinositol 3-kinase. PI(3,4,5)P3 recruits the Ser/Thr kinases phosphatidylinositol-dependent protein kinase1 (PDK1) and AKT to the plasma

membrane, where AKT is activated by PDK1-mediated phosphorylation. AKT phosphorylates many proteins, including glycogen synthase kinase 3b (GSK3B)in liver,AS160(GLUT4 translocation) and forkhead box O transcription factors(regulation of gene expression) (Fig.1.1).



Adopted from: Taguchi A and White FM2008 Insulin –like signaling, nutrient homeostasis, and life span. Annual Review of physiology70, 191-212

Figure (1.1) insulin signaling pathways regulating GLUT4 translocation in mammalian skeletal muscle. Insulin activates tyrosine kinase activity of insulin receptor(IR) by binding with α-subunit of IR. Activated IR phosphorylates itself and insulin receptor substrate-1(IRS-1). Phosphorylated IRS-1 binds to PI3-kinase, which is recruited to plasma membrane and converts phosphatidylinositol-4,5-biphosphate(PIP2) to phosphatidylinositol-3,4,5-triphosphate(PIP3). Increased PIP3 recruits phosphatidylinositol-dependent protein kinase-1(PDK1) and AKT to plasma membrane where AKT is activated by PDK1-mediated phosphorylation. Activated AKT phosphorylates AS160/TBC1D1,which inhibits its Rab GTPase-activating protein(GAP) activity towards particular Rab isoform(s). inhibition of GAP increases conversion of less active GDP-loaded Rab to more active GTP-loaded Rab. Increased active GTP-loaded Rab then allows GLUT4 storage vesicles to move, dock, and fuse with plasma membrane. GLUT4 vesicles bind to plasma membrane via interaction of VAMP2 with target-SNARE complexes.

1.4 Translocation of glucose transporter to plasma membrane:

The transport of glucose into the skeletal muscle cells is the rate-limiting step in whole body glucose metabolism under normoglycemic condition(Ren et al., 1995). Glucose enters the cell by facilitated diffusion mediated via a group of structurally related glucose transporter proteins (GLUTs). At least 12 GLUTs have been described(Joost et al., 2002). In skeletal muscle and adipose tissue, GLUT1 mediates basal glucose transport, whereas GLUT4 is responsible for insulin-mediated glucose uptake (Tordjman et al., 1989). The stimulation of GLUT4 translocation and glucose

uptake by insulin is a complex process. The necessity of the IRS-PI3K-AKT-AS160 axis in insulin stimulated glucose uptake is well documented (Björnholm and Zierath, 2005).

1.5 Type1 diabetes mellitus:

On the basis of epidemiology and etiology, type1 diabetes represents around 10%of all cases of the diabetes, affecting approximately 20 million people worldwide(Garcia et al., 2001).

Although type1 diabetes affects all age groups, the majority of individuals are diagnosed either at around the age of 4 to 5 years, or in their teens and early adulthood(Bloom et al., 1975).

Type1 is present in patients who have little or no endogenous insulin secretory capacity and who therefore require insulin therapy for survival. The two main forms of clinical type1 diabetes are type1a (about 90% of type1 cases in Europe)which is thought to be due to immunological destruction of pancreatic β cells resulting in insulin deficiency; and type1b(idiopathic, about 10% of type1 diabetes), in which there is no evidence of autoimmunity. Type1a is characterized by the presence of islet cell antibody(ICA), anti-glutamic acid decarboxylate(anti-GAD), IA-2 or insulin antibodies that identify the autoimmune process with β-cell destruction (Zimmet et al., 2004, Epstein et al., 1994). autoimmune diseases such as Grave's disease, Hashimoto's thyroiditis and Addison's disease may be associated with type1 diabetes mellitus(Betterle et al., 1984). There is no known etiological basis for type1b diabetes mellitus(McLarty et al., 1990). This form is more prevalent among individuals of African and Asian origin(Ahren and Corrigan, 1985).

1.6 Type2 diabetes mellitus:

Type2 diabetes is a heterogenous disorder caused by a combination of genetic factors related to impaired insulin secretion, insulin resistance and environmental factors such as obesity, over eating, lack of exercise, and stress as well as aging (Kaku et al., 2010).

Type2 diabetes is the common form of idiopathic diabetes and is characterized by a lack of the need for insulin to prevent ketoacidosis. It is not an autoimmune disorder and the susceptible genes that predispose to NIDDM have not been identified in most patients. This could be due to the heterogeneity of the genes responsible for the susceptibility to NIDDM.

Given the existence of insulin resistance and predisposing genetic background, there occurs elevation of glucose levels, which lead to progressively decreases of insulin secretion, insulin gene expression, insulin promoter activity (PDX-1 and MafA) (Poitout, 2008, Khaldi et al., 2004).

1.6.1 Current understanding of T2DM:

1.6.1.1 Skeletal muscle

Since skeletal muscle accounts for~75% of whole body insulin-stimulated glucose uptake, defects in this tissue play a major role in the glucose homeostasis in patients with T2DM (Björnholm and Zierath, 2005). Insulin receptor tyrosine phosphorylation appears to be normal or reduced in nonobese T2DM (Zierath et al., 2000). Type2 diabetic subjects have impaired insulin-stimulated tyrosine phosphorylation of IRS1in skeletal muscle. A similar impairment is observed at the level of PI3K in type2 diabetic muscle(Zierath et al., 2000). The dysregulation of the insulin receptor or IRS constitutes a common feature of insulin resistance.

Patients with T2DM are characterized by a decreased fat oxidative capacity and high levels of circulating free fat fatty acids (Kelley and Simoneau, 1994, Blaak et al., 2000). The latter is known to cause insulin resistance, particularly in skeletal muscle, by reducing insulin –stimulated glucose uptake, most likely via accumulation of lipid inside the muscle cell (Boden, 1999, Santomauro et al., 1999).

1.6.1.2 Adipose tissue

GLUT4 expression is down-regulated in adipose tissue in patients with T2DM (Epstein et al., 1999). Given that skeletal muscle is the major site for glucose disposal, the hyperglycemia associated with T2DM cannot be explained by the decreased uptake of glucose into adipose tissue due to downregulation of GLUT4 in adipocytes.

Furthermore, adipocyte-selective knockout of GLUT4 (adipose-Glut4K/K) in mice resulted in systemic insulin resistance similar to that induced by muscle-selective Glut4 knockout mice (Abel et al., 2001, Zisman et al., 2000). These studies indicate that adipocyte GLUT4 deficiency may result in generation of circulating factors that are responsible for crossorgan communication.

1.6.1.3 Pancreatic β-cells

It is estimated that at diagnosis of T2DM, Beta cell function is already reduced by 50-60% and that this reduction of beta-cell function seems to start with 10-12 years before the appearance of hyperglycemia (Group, 1995, Levy et al., 1998).

Chronic exposure of beta-cells to hyperglycemia can also induce beta-cells apoptosis by increasing proapoptotic genes expression(Bad, Bid, Bik) while antiapoptotic gene expression Bcl-2remains unaffected (Patanè et al., 2002).

There is a strong relationship between glucotoxicity and lipotoxicity. Thus, hyperglycemia increases malonyl-CoA levels, leading to the inhibition of carnitine palmitoyltransferase-1 and subsequently to decreased oxidation of fatty acids and lipotoxicity (Poitout and Robertson, 2008).

Increased fatty acids pancreas leads to intrapancreatic accumulation of triglycerides (Gastaldelli, 2011). Earlier studies showed that the intrapancreatic fat is associated with beta-cell dysfunction and that sustained negative energy balance induces restoration of Beta-cell function (Lim et al., 2011).

In T2DM, the typical Beta-cell functional alterations are represented by:

- Change of threshold for insulin secretion triggering with relatively selective loss of responsivity to glucose compared to other insulin secretagogues like arginine or glibenclamide.
- Alteration of insulin secretion oscillatory patterns.
- Reduced or absent first phase insulin secretion.
- Prolongation of second phase of insulin secretion.
- Gradual, time-dependent irreversible damage to cellular components of insulin production (Del Prato et al., 2002, Kahn, 2003, Marchetti et al., 2006).

1.6.1.4 Liver

Liver is the major organ with the ability to consume, store, and produce glucose and lipids. Hepatic glucose metabolism includes the formation of glycogen(short term energy storage), generation glucose from nonsugar carbon substrates and intracellular energy supply via glycolysis (Klover and Mooney, 2004). Fatty acid oxidation, de novo synthesis of fatty acids, cholesterol and bile acid synthesis, as well as lipoprotein assembly are the essential processes in lipid homeostasis under physiological conditions(Raddatz and Ramadori, 2007). Consequently, the liver is a key target for the anabolic hormone insulin and its catabolic counterpart glucagon. Insulin is released from the pancreatic β-cells in response to increased blood glucose concentrations, and this is amplified in the presence of FFAs. Impaired insulin sensitivity and dysregulated insulin action in the liver contributes significantly to the pathogenesis of T2DM(Fritsche et al., 2008).

IRS-1 and IRS-2 are complementary key players in the regulation of hepatic insulin signaling and expression of genes involved in gluconeogenesis, glycogen synthesis, and lipid metabolism(Fritsche et al.,2008). Dysfunction of IRS proteins initially leads to postprandial hyperglycemia, increased hepatic glucose production, and dysregulated lipid synthesis, and is considered as a major pathophysiological mechanism for the development for insulin resistance and T2DM(Taniguchi et al., 2005, Dong et al., 2006, Simmgen et al., 2006).

1.6.1.5 Multiorgan disease

GLUT4 inactivation limited to white adipose tissue resulted in insulin resistance, glucose intolerance, and evenT2DM with impairment in whole body glucose uptake markedly out of proportion to the expected contribution of white adipose tissue(Minokoshi et al., 2003). This isolated defect in white adipose tissue resulted in defective insulin action in both muscle and liver. These studies suggest a central and previously under-appreciated role of adipocytes in the pathogenesis of T2DM. However, insulin receptor inactivation in the liver also resulted in hyperinsulinemia, hepatic insulin resistance, and peripheral insulin resistance(Michael et al., 2000, Baudry et al., 2002, Mauvais-Jarvis et al., 2002, Fisher and Kahn, 2003). These studies suggest considerable crosstalk among organ system, as well as an important

role for insulin signaling pathways in the β-cell(Accili, 2004). The central nerve system(CNS) has been shown to have an essential role in regulating glucose metabolism by sensing integrating information from neural, hormonal, and nutrient signals, and then modulating glucose output in the liver and glucose uptake in peripheral tissue(Sandoval et al., 2009). Data from animal experiments have been documented to show that overeating and obesity dampen the ability of the CNS to sense and respond to the information, whereas selective CNS interventions decrease insulin resistance and blood glucose levels(Sandoval et al.,2009). Thus, T2DM is the final outcome of a multiorgan disease.

1.6.1.6 Mitochondrial and reactive oxygen species

Several lines of evidence support the association of oxidative stress markers with diabetes(Urakawa et al., 2003, Furukawa et al., 2004, Lin et al., 2005). Recent data showed that treatment of 3T3-L1 adipocyte with either TNFα or dexamethasone increased the ROS level and resulted in decreased insulin action. Antioxidant molecules or transgenes encoding ROS scavenging enzymes both ameliorated the insulin resistance of TNFα- or dexamethasone-treated 3T3-L1 adipocytes to varying degrees. Furthermore, antioxidant molecules improved insulin sensitivity and glucose homeostasis in ob/ob mice (Houstis et al., 2006).

Supportive studies in humans have suggested a role for defective mitochondrial fatty acid oxidation, mitochondrial dysfunction, and reduced numbers of skeletal muscle mitochondria in T2DM pathogenesis, and have suggested that increased intramyocellular lipid content was associated with defects in mitochondrial activity (Maechler and Wollheim, 2001, Petersen et al., 2004, Lowell and Shulman, 2005, Morino et al., 2005). There is 38% less mitochondrial density in muscle in insulin resistance individuals compared with controls (Morino et al.,2005). The decreased mitochondrial fatty acid oxidation, caused by either mitochondrial dysfunction and/or reduced mitochondrial numbers, produces increased levels of intracellular fatty acyl CoA and diacylglycerol. These molecules activate a novel protein kinase C, which in turn activates a serine kinase cascade, leading to increased serine phosphorylation of IRS1, serine phosphorylation blocks IRS1 tyrosine phosphorylation and inhibits downstream signaling, including recruitment of GLUT4 to the plasma membrane and insulin-mediated glucose uptake in skeletal muscle (Lowell and Shulman, 2005).

1.6.1.7 Obesity and physical inactivity

About 80% of T2DM is associated with obesity and sedentary life styles(Venables and Jeukendrup, 2009). It is well accepted that obesity and physical inactivity are risk factors for the development of T2DM (Weinstein et al., 2004).

In obesity, the disparity between uptake of fatty acids into skeletal muscle and oxidation results in excessive accumulation of triacylglycerol and fatty acid metabolites such as long-chain acyl-CoAs, diacylglycerols, and ceramides in the sarcoplasm of skeletal muscle (Venables and Jeukendrup, 2009). An increase in skeletal muscle diacylglycerol content in human and animal models of insulin resistance activates specific isoforms of protein kinase C, leading to inhibition of insulin signal through serine phosphorylation of IRS1(Itani et al., 2002, Yu et al., 2002). For obesity and insulin resistance to be associated with type2 diabetes, b-cells must be unable to compensate fully for decreased insulin sensitivity(Kahn et al., 2006). Clinical trials have also shown that addition of physical activity and dietary modification can reduce the incidence of T2DM (Venables and Jeukendrup, 2009). Recent data have indicated that elevations in mitochondrial oxidative capacity following an acute bout of exercise can increase insulin-stimulated glucose uptake (Thyfault et al., 2007).

1.6.1.8 Genetic analysis

There is ample evidence that T2DM has strong genetic component, which includes monogenic disease such as MODY1-6 (described to date) under 25 years of age and polygenic disease such as T2DM(Majithia and Florez, 2009). There were detected several genetic variants of genes that confer risk of diabetes by interfering with next three mechanisms

- Reduction of insulin secretion.
- Impairment of incretin release.
- Impaired proinsulin-to-insulin conversion.

The most important so far type2 diabetes risk gene, TCF7L2, interferes with all three mechanisms. TCF7L2 encodes for the transcription factor TCF7L2, which induces the expression of a number of genes including the insulin gene (Loder et al., 2008), the

gene coding for intestinal proglucagon (Yi et al., 2005), genes coding for proprotein convertases1 and 2 (Loos et al., 2007) and for proteins important in insulin exocytosis and genes critical for beta-cell proliferation (Rulifson et al., 2007).

1.7 Glutathione-s-transferases(GSTs):

The glutathione-s-transferases are a multi-gene family of enzymes involved in the detoxification, and, in a few instances, activation of a wide variety of chemicals.

1.7.1 Types of Glutathione-s-transferases:

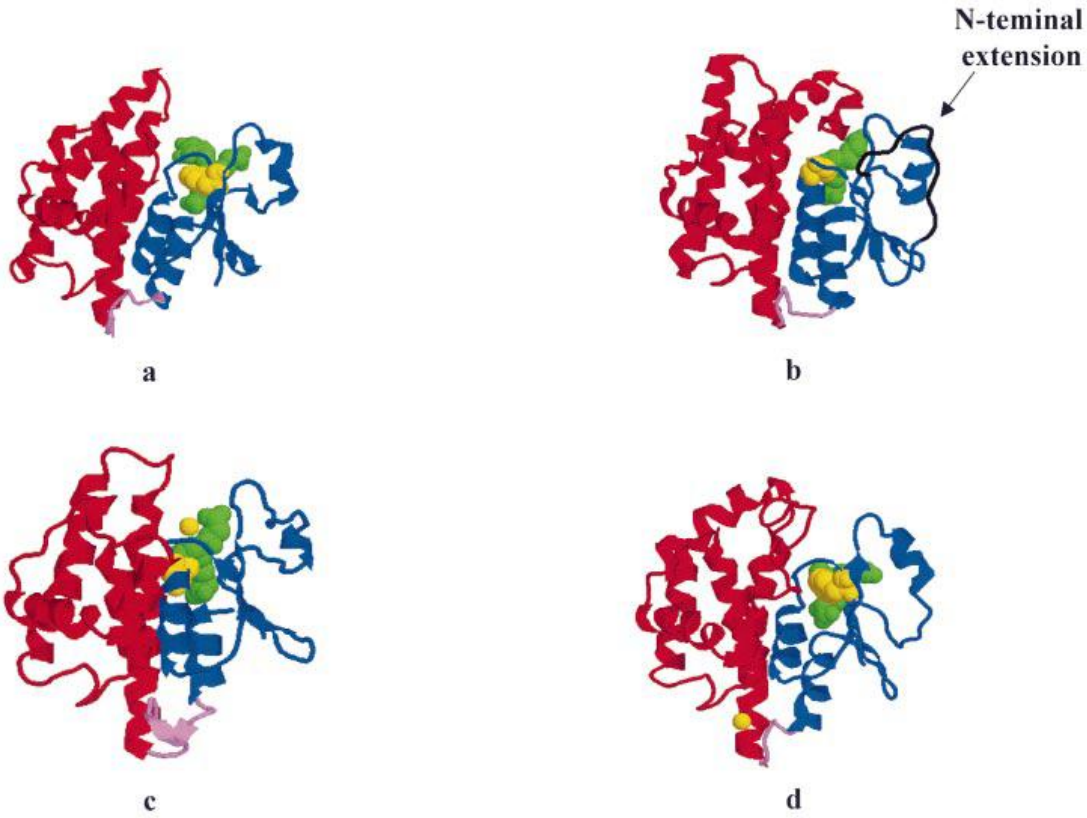
Most GSTs exist as soluble enzymes, although a small family of microsomal GSTs has been characterized(Jakobsson et al., 1996), and mitochondrial GST, referred to as GST Kappa, has also been identified (PEMBLE et al., 1996). In humans, the soluble GSTs are collectively expressed in rather large amounts, constituting as much as 4% of total soluble protein in the liver.

1.7.2 Structure of soluble Glutathione-S-transferases:

The soluble forms of GSTs exist as dimeric proteins, with subunit molecular weights of approximately 25 KDa. Each subunit of dimeric enzyme has an active site composed of 2 distinct functional regions: a hydrophilic G-site, which binds the physiological substrate glutathione, and an adjacent H-site which provides a hydrophobic environment for the binding of structurally diverse electrophilic substrates (Armstrong, 1997).The G-site is highly conserved between all GSTs due to its high specificity for GSH, whereas the H-site can be quite divergent between different GSTs, and exhibits broad and variable substrate binding specificity.

Despite the low overall level of sequence identity across the classes, all the structures follow a similar canonical fold, with each subunit consisting of two distinct domains(figure3.1)(Wilce and Parker, 1994). The N-terminal domain1(approx. residues 1-80) adopts a topology similar to that of the thioredoxin fold(Wilce and Parker, 1994, Martin, 1995), consisting of four β-sheets with three flanking three α-helices(figue2.1). This fold occurs in several proteins of limited sequence identity from other enzyme families, which appear to have evolved to bind cysteine or GSH. The fold consists of distinct N-terminal and C-terminal motifs which have a βαβ and

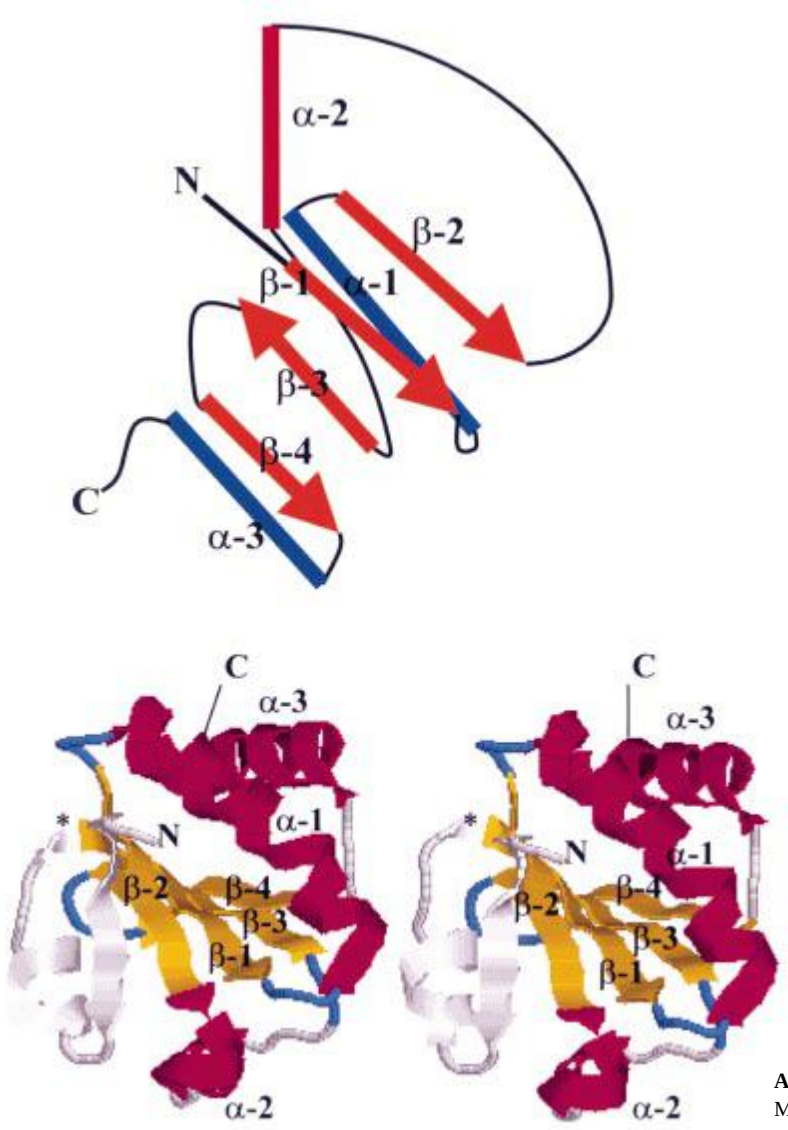
ββα arrangement respectively, and which are linked by an α-helix(α-2 in figure3.1). The former begins with an N-terminal β-strand(β1), followed by an α-helix(α-1). And then a second β-strand (β-2) which is parallel to β-1. A loop region leads into a second α-helix(α-2), which connects with the c-terminal motif. This motif consists of two sequential β-strands (β3-and β-4),which are antiparallel which are followed by a third α-helix(α-3) at the c-terminus of the fold. The four β- sheets are essentially in the same plane, with two helices (α-1 and α-3) below this plane and α-2 above it, facing the solvent. The loop that connects α-2 to β-3 features a characteristic proline residue which is in the less favoured *cis* conformation and is highly conserved in all GSTs. This is referred to as the *cis*-Pro loop which, while playing no direct role in catalysis, appears to be important in maintaining the protein in a catalytically competent structure (Allocati et al., 1999). In GSTs, domain 1 is highly conserved and provides most of the GSH binding site. It is connected to domain 2 by a short linker sequence, as shown in figure(2.1) (Dirr et al., 1994). Domain 2 (approx. residues 87-210) begins at the C-terminus of the linker sequence, and consists of five α-helices in the case of the Pi and Mu classes (Ji et al., 1992)and six α-helices in the case of Alpha classes(Sinning et al., 1993) (the number of helices in domain 2 varies widely between classes). The C-terminal domain is less similar between the three classes than the N-terminal domain (Dirr et al., 1994, Wilce and Parker, 1994). It contributes most of the residues that interact with the hydrophobic second substrate, as well as contributing a highly conserved aspartic acid residue (occurring in helix α-4) to the GSH binding site. Differences in the C-terminal domain may be responsible for differences in substrate specificity between the three classes (Wilce and Parker, 1994).



Adopted from: Sheehan D, Meade G, Vivienne M.F.& Catriona A.D.2001 Structure, function and evolution of glutathione transferases. Biochem. J. 360,1-16

Figure(2.1) Domain structure of GST subunits

Three-dimensional structures of individual GST subunits are shown. The N-terminal domain1 is coloured blue, while the C-terminal domain2 is coloured red. Catalytically essential residues(tyrosine in **a** and **d;** cysteine in **b** and **c**) are coloured yellow and presented in space-filling mode, while ligands with which the protein was co-crystallized are shown in green. Linker strands connecting the two domains are shown in violet.



Adopted from: Sheehan D, Meade G, Vivienne M.F.& Catriona A.D.2001 Structure, function and evolution of glutathione transferases. Biochem. J. 360,1-16

Figure(3.1)The thioredoxin fold

A schematic diagram representing the thioredoxin fold is shown above a depiction of the thioredoxin dimer. In the diagram, α-helices are shown as cylinders, while β-sheets are shown as orange arrows. The four β-sheets are essentially co-planar, with one helix(α-2) shown in red above this plane and the other two α-helices(α1-α3) shown in blue below the plane. The cis-Pro loop links α-2 to β-3. In GSTs, domain 2 is connected to the c-terminus by a short linker peptide. In thioredoxin itself, β-sheets are coloured yellow, while α-helices are magenta. The thioredoxin fold has an extra β-sheet and α-helix at the N-terminus(residues 1-21) ending at the point donated by* where the fold proper begins.

1.7.3 Nomenclature of soluble Glutathione-S-transferaes:

The soluble GSTs are divided into 4 main classes, alpha(A), mu(M), pi(P), and theta(T)(Harris et al., 1998, Hayes and Pulford, 1995) although a new form, zeta[GSTZ1;(BOARD et al., 1997)], has been identified recently. A sigma class GST has been described in certain invertebrate species(Ji et al., 1992) and also in rats, after initial identification as prostaglandin D synthase(Kanaoka et al., 1997). A standardization nomenclature for human GSTs was proposed in 1992(Mannervik et al., 1992) and was extended to include other species in 1995(Hayes and Pulford, 1995)This nomenclature identifies each GST by species using: lower case letter(s) preceding "GST"(h for human, r for rat, m for mouse, etc.), followed by an upper case letter denoting the class (A, M, P, T,Z), then an Arabic numeral denoting subfamily (1,2,3….), and in a few instances, a lower case denoting allelic variants of the same gene (a,b,c….). The functional GST enzymes exist as dimeric proteins, and only subunits within the same class can form heterodimers(e.g., alpha subunits can dimerize with other alpha subunit types, but not with mu or pi subunits). Therefore, the nomenclature for the enzyme identifies each of the 2 subfamily Arabic numbers, separated by a hyphen. Thus, *hGSTM1a-1a* refers to the homodimeric protein in which both subunits are from the specific allelic variant "a" of the human mu class protein from subfamily1; *rGSTA1-2* refers to a rat heterodimeric alpha class protein

1.7.4 Gene Variants

GSTM1 which locates on chromosomes 1p13.3 is polymorphically expressed, and three alleles have been identified:*GSTM1-0, GSTM1a* and *GSTM1b(Rebbeck, 1997)*. There is no evidence of functional differences between *GSTM1a* and *GSTM1b*, which differ by a substitution in one base pair (Cotton et al., 2000). The *GSTT1* locus which locates on chromosome 22q11.2 has a single allele that is either present or absent (deleted). Two functionally different genotypes at the *GSTT1* locus have been described. The first, *GSTT1-0*, is a homozygous deletion. The second, *GSTT1-1*, comprises genotypes with one or two functional alleles present. *GSTP1* which locates on chromosome 11q13 has three variant alleles. *GSTP1a* and *GSTP1b* differ by an A to G substitution in exon 5 at codon 105 that exchanges isoleucine for valine. *GSTP1c* varies from the "a" allele by the substitution of valine for alanine at codon 114.

Individuals with homozygous deletion of *GSTM* or *GSTT* have reduced or no glutathione-s-transferase activity and therefore may be unable to eliminate electrophilic carcinogens as efficiently. This may increase the risk of somatic mutations leading to tumor formation. Evidence is lacking on whether heterozygosity in either *GSTM1* or *GSTT1* affects gene function(Cotton et al., 2000). An analysis of functional differences in the *GSTP1* variant proteins was performed by evaluating the ability of recombinant *GSTP1* proteins to catalyze the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene, a universal GST substrate. Results showed catalytic activity was 3-to4-fold higher for the *GSTP1a* variant than for either *GSTP1b* or *GSTP1c(Ali-Osman et al., 1997).* This indicates that enzyme activity is significantly reduced in individuals who carry the valine allele. Studies have been conducted on all continents with the exception of Africa and South America; only two studies have been conducted in the United States. The frequency of the homozygous wildtype genotype at exon5 ranges from 39% to 59% in Whites, 52% to 93% in Asians, and 35%in the single study involving African-Americans. The heterozygous genotype ranged from 30% to 50% among Whites, 24% to 44% among Asians, and 46% among African-Americans. The homozygous mutant genotype ranged from 4% to 16% among Whites, 4% to 5% among Asians, and 19% for African-Americans. For exon6 polymorphism, the frequency of the homozygous wild type genotype ranged from 75% to 85% among Whites, 98% to 100% among Asians, and 95% among African-Americans. Heterozygous genotypes ranged from 15% to 25% among Whites, 0% to 2% among Asians, and 5% among Africans-Americans. The homozygous mutant genotype was only found among persons from India(2%).

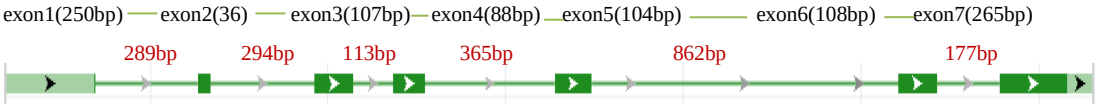


Figure (4.1) diagram of GSTP1 gene

1.7.5 Catalytic Function

Types of reactions

GSTs catalyze the general reaction: GSH+R-X ⟶ GSR+HX. The function of the enzyme is to (1) bring the substrate into close proximity with glutathione(GSH) by

binding both GSH and the electrophilic substrate to the active site of the protein, and (2) activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH on the electrophilic substrate (R-X) (Armstrong, 1997). The electrophilic functional center of the substrates can be a carbon, nitrogen or sulfur. The formation of a thioether bond between the cysteine residue of GSH and the electrophile usually results in a less reactive and more water soluble product, and thus GSTs are usually detoxification reactions.

Typical substrates

A large number of diverse chemicals serve as substrates for GSTs. Many epoxide carcinogens are detoxified by GSTs, and differences in expression of specific isoforms can be an important determinant of target organ and species sensitivity. For example, mice are remarkably resistant to the hepatocarcinogenic effects of aflatoxin B1(AFB) because they constitutively express mGSTA3-3, which has high activity toward the reactive AFB *exo*-epoxide(Eaton and Gallagher, 1994). The carcinogenic epoxide of benzo(a)pyrene (BPDE) is efficiently detoxified by GSTs, with especially high and selective activity exhibited by hGSTP1-1 (Sundberg et al., 1998). The trans isomer of stilbene oxide(TSO) is uniquely conjugated by hGSTM1-1, and thus this epoxide substrate serves as a selective marker for this polymorphic mu class GST (Seidegård et al., 1989). Chlorinated nitrobenzenes (1-chloro-2,4-dinitrobenzene, CDNB; 1,2-dichloro-4-nitrobenzene, DCNB) have long served as standard substrates for nearly all GSTs. However, theta class GSTs do not catalyze these reactions, and the specific activities toward CDNB and DCNB can vary greatly between different isoforms. The diuretic ethacrynic acid (ECA) is both a good substrate and a good inhibitor of certain GSTs (Awasthi et al., 1993, Ploemen et al., 1993). It has been used as a selective marker for Pi class GSTs, although certain other GSTs also exhibit relatively high ECA activity. The glutathione conjugates formed are generally unstable and rearrange or dissociate to reactive forms such as episulfonium ions (ethylene dibromide) or formaldehyde (dichloromethane). One important role of GSTs is in detoxification of endogenous products of lipid peroxidation such as 4-hydroxy-2-nonenal.

Human GSTA4-4 has unusually high activity toward this substrate, and may play an important physiological role in protecting against oxidative stress induced by endogenous lipid peroxides(HUBATSCH et al., 1998).

Inhibitors of GSTs

GST inhibitors that are relatively non-toxic, isoenzyme specific, and active in vivo have not yet been developed. Despite a considerable effort, so far the only in vivo active inhibitors of GSTs are ethacrynic acid and a number of glutathione derived structures (Morgan et al., 1996, Mulder and Ouwerkerk-Mahadevan, 1997). A number of inhibitors have been reported that are suitable for in vitro studies. These include ethacrynic acid, the antibiotic calvatic, curcumin, disulfiram, and organotin compounds

1.7.6 Tissue specific and developmental expression of human GSTs

Regulation of GST expression differs among tissues, such that not all GST isoforms are expressed in every tissue. Furthermore, some GSTs are polymorphic, and in the case of both *GSTM1* and *GSTT1*, gene deletion**s** are quite prevalent in the human population. Thus, individuals homozygous for the *GSTM1* deletion polymorphism (approx. 50% of the population) will lack expression of this enzyme in any tissue in the body. Due to such interindiviual genetic differences, the complex, tissue-specific expression pattern of GSTs, and modulation by diet and xenobiotics it is difficult to predict accurately the extent of expression of any GST gene in a given tissue. However, immunoblot and HPLC-electrospray-mass spectrometry subunit of analyses GST isoforms have provided some information on relative expression of different classes of GSTs in human(Hayes and Pulford, 1995, Listowsky et al., 1998). Alpha class GSTs are, in general, relatively high expressed in liver, kidney, testis, but not in lung, whereas GSTP1-1 is expressed in relatively high levels in brain and lung, but not in liver. Depending on the specific sub-family, mu class GSTs are expressed to different extents in different tissues. *hGSTM2*, for example, is expressed in highest levels in the brain and hardly at all in the liver, whereas *hGSTM1* is expressed at highest levels in the liver in those individuals who carry 1 or 2 functional alleles. The testes express a variety of GSTs, but *GSTM3* is expressed almost uniquelyin this tissue(Listowsky et al., 1998). Theta class GSTs are expressed predominately in liver

and kidney, to a lesser extent in other organs, and only at relatively low levels in lung (SHERRATT et al., 1997).

1.8 Role of ROS in the progression of pancreatic β-cell dysfunction in Type 2 Diabetes

Acute exposure of β-cells to a high glucose concentration stimulates insulin gene expression, but chronic exposure has various adverse effects on various β-cell function. However, chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. This process is called β-cell glucose toxicity which is often observed under diabetic conditions. In the diabetic state, hyperglycemia per se and subsequent production of ROS decrease insulin gene expression and secretion and finally bring about apoptosis. It has been shown that the loss of insulin gene expression is accompanied by decreased expression and/or DNA binding activity of transcription factors: pancreatic and duodenal homeobox-1(PDX-1) (Kaneto et al., 1999, Tanaka et al., 2002) and MafA (Poitout and Robertson, 2002, Moran et al., 1997). After chronic exposure to a high glucose concentration, expression and/or DNA binding activities of these two transcription factors are reduced. It is noted here that PDX-1 plays a crucial role in pancreas development, β-cell differenation, induction of surrogate β-cells, and maintenance of mature β-cell function(Ohlsson et al., 1993, Kaneto et al., 2008) and that MafA is a recently isolated β-cell specific transcription factor and functions as a potent activator of insulin gene transcription. In addition, β-cells are rather vulnerable to ROS due to the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase (Olbrot et al., 2002). Therefore, it is likely that ROS are involved in β-cell deterioration found in diabetes.

It has been suggested that activation of the c-Jun N-terminal kinase (JNK) pathway is involved in pancreatic β-cell dysfunction found in type 2 diabetes. It was reported that activation of the JNK pathway is involved in reduction of insulin gene expression by ROS and that suppression of the JNK pathway can protected β-cells from ROS(Olbrot et al., 2002).

1.9 Role of ROS in the progression of insulin resistance in type 2 diabetes

The hallmark of type 2 diabetes is insulin resistance as well as pancreatic β-cell dysfunction. Under diabetic conditions, various insulin target tissues such as the liver, muscle, and fat become resistant to insulin. The pathophysiology of insulin resistance involves a complex network of insulin signaling pathways. After insulin binds to insulin receptor on cell surface, insulin receptor and its substrates are phosphorylated, which leads to activation of various insulin signaling pathways (Shulman, 2000, Kahn and Flier, 2000). It has been shown that ROS are involved in the progression of insulin resistance as well as pancreatic β-cell dysfunction(Evans et al., 2002). Indeed, it was previously reported that ROS disrupted insulin-induced cellular redistribution of insulin receptor substrate-1(IRS-1) and phosphatidylinositol 3-kinase (PI3-k), and thus impaired insulin-induced GLUT4 translocation in 3T3-L1 adipocyte (Tirosh et al., 1999). It was also reported that treatment with antioxidants prevented hyperglycemia-induced insulin resistance in vivo (Haber et al., 2003). Finally, it has been suggested that activation of the JNK pathway is involved in insulin resistance as well as pancreatic β-cell dysfunction found in diabetes (Jacob et al., 1999).

1.10 Role of ROS in the progression of Atherosclerosis

Atherosclerosis is often observed as a macroangiopathy under diabetic conditions. Indeed, it has been reported that increase of intima-media thickness(IMT) in carotid artery, an index of the progression of atherosclerosis, is often observed in diabetic patients (Kawamori et al., 1992).

It has been shown that ROS are induced in endothelial cells under diabetic conditions. There are several sources of reactive oxygen species(ROS) in cells such as the nonenzymatic glycosylation reaction, the electron transport chain in mitochondria, and membrane-bound NADPH oxidase. It has been shown that membrane-bound NADPH oxidase is the one of the major sources of ROS in the vasculature and that NADPH oxidase-derived ROS play a crucial role in the of atherosclerosis. NADPH oxidase is composed of the membrane-bound subunits gp91 phox(Nox2)/Nox1/Nox4 and p22 phox, and the catalytic site of the oxidase and cytosolic components p47 phox and p67 phox. In vasculature cells such as endothelial and smooth muscle cells,

Nox1 and Nox4, rather than gp91 phox, are abundantly expressed. NADPH oxidase is activated by various factors such as AGEs, insulin, and angiotensin II; all of which are possibly induced under diabetic conditions(Ushio-Fukai et al., 1996). In addition, it was shown that high glucose stimulates ROS production through the activation of NADPH oxidase (Guzik et al., 2002) and that the p22 phox was significantly increased in rat and human diabetic arteries (Arai et al., 1997). Therefore, it is possible that such increased expression of p22 phox contributes to the development of atherosclerosis. Increased ROS are involved in the development of atherosclerosis in various aspects. First, endothelial dysfunction is an early key event in atherosclerosis (Ushio-Fukai et al., 1996, Madamanchi et al., 2005). It has been thought that ROS are involved in the progression of endothelial cell dysfunction, which is accompanied by inactivation of endothelial nitric oxide synthase (eNOS) and decrease of nitric oxide (NO) levels (Madamanchi et al., 2005). Second, ROS also induce expression of adhesion molecules such as intercellular adhesion molecule-1(ICAM-1) and vascular adhesion molecule-1(VCAM-1), which facilitates inflammatory cell recruitment and lipid deposition in the intimal layer. The subsequent ingestion of excess oxidized low density lipoprotein (LDL) particles by macrophages and monocytes leads to release of various inflammatory cytokines and growth factors. Finally, proliferation of vascular smooth muscle cells (VSMCs)is a key step in the development of atherosclerosis. It has been suggested that ROS regulate expression of various growth factors and several growth-related protooncogens such as c-Myc, c-Fos and c-Jun (Delafontaine and Ku, 1997). The JNK pathway is known to be activated by ROS in VSMC (Yoshizumi et al., 2000), and activation of the JNK pathway is likely involved in the progression of atherosclerosis. It is known that the JNK pathway plays an important role in the initiation of cellular response, including cellular gene expression, growth, migration, or apoptosis.

1.11 Aim of study

Type 2 diabetes mellitus (T2DM) is a significant global health problem. Reactive oxygen species (ROS) production induced by chronic hyperglycemia is implicated as a potential molecular mechanism behind diabetic vascular complication. The ROS activates protein kinase C(PKC) and increased the production of advanced glycation end products(AGEs), leading to superoxide generation which trigger atherosclerosis. Pancreatic β-cells express low levels of antioxidant enzymes and become sensitive to cytotoxic stress that leads to higher risk of oxidative damage.

A GSTP1 variant with A to G transition in exon 5 at codon 105 leads to Ile105Val amino acid substitution, which reduces the ability to conjugate reactive electrophiles with glutathione and may therefore sensitize cells to free radical-mediated damage.

Studies have so far reported contradictory results regarding any association between GSTP1 gene polymorphism and T2DM. Thus, we conducted a case-control study in the Libyan population with age-and sex-matched T2DM to evaluate whether the GSTP1 variants modulate the risk of T2DM patients and to estimate glycemic and lipid parameters for T2DM patients and normal one.

2 -Subjects and Methods

2.1 Subjects

A total of 67 Libyan patients with type 2 DM (31 females and 36 males) were recruited from the Benghazi Center for Diagnosis and Treatment of Diabetes, and apparently healthy age and sex-matched individuals(10 females and 16males) were recruited from the high institute of Medical Profession-Benghazi, to serve as controls.

The diagnosis of diabetes was based on previous history of diabetes based on the American Diabetes Association Criteria 2006(i.e. A1c≥ 6.5%, or fasting plasma blood glucose≥126mg/dl, or 2-h plasma glucose≥200mg/dl during an oral glucose tolerance test.

Clinical information and medical history were obtained through patients' interviews. Face-to-face interview that included variables such as age, sex, and onset of diabetes.

The height and weight were measured and obesity was defined as body mass index(BMI) of ≥ 30kg/m2 where BMI was calculated by dividing the weight in kilograms on height in meters squared. Blood pressure was measured using mercury sphygmomanometer with subjects in a seated position.

All patients were presented-stable metabolic conditions.

Patients were presenting any disease that could affect their metabolic status and the parameters studies such as nephritic syndrome, acute or chronic renal failure, liver disease, thyroid disorders, acute infections, stroke, gout diabetic ketoacidosis and non-ketotic hyperosmolar syndrome were excluded.

The history of medication was recorded.

The control group consisted of healthy subjects who were not suffering from any acute infection, they were non-smoker, and non-over weight, they had no history of familial hypercholesterolemia or DM.

2.2 Methods:

2.2.1 Blood collection:

Venous blood samples were drawn from all the participants after at least 10 hours fasting. Blood was collected in EDTA and plain tubes, and sera were separated from plain tubes and assays were performed. The whole blood was stored at 2-8C, and extracted DNA and analyzed for HbA1c within two days. Sera were analyzed for triacylglycerol, total cholesterol, blood glucose, HDL and LDL.

2.2.2 Instruments:

The measurements of glucose, HbA1c, triacylglycerol(TAG), high density lipoprotein(HDL), and low density lipoprotein LDL were done using the standard procedures and available commercial kits in a fully automated system Beckman Glucose Analyzer, I-Chroma, and ChemWell.

2.2.3 Estimation of fasting blood glucose(FBG):

Test principle:

The Beckman Glucose Analyzer is an instrument for determining glucose in plasma, serum and urine. Glucose was determined by means of the oxygen rate method employing a Beckman oxygen sensor. An enzyme reagent containing glucose oxidase converts the glucose in the sample combined with dissolved oxygen from the solution according to the reaction:

$$\text{Glucose} + O_2 \xrightarrow[H_2O]{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

The rate of oxygen consumption is determined by an oxygen sensor coupled to an electronic system. This is directly proportional to glucose concentration in the sample.

2.2.4 Estimation of HbA1c:

Test principle:

The method employed in the *i*-CHROMA HbA1c is based on the fluorescence immunoassay technology, specifically the competition immune-detection method. Whole blood is added to the mixture of hemolysis buffer and detection buffer, which resultes in hemolysis of red blood cells. The mixture containing HAb1c from the hemolyzed red blood cells and fluorescence-labeled HbA1c peptides from detection buffer is loaded onto the sample well of the cartridge. The mixture then migrates through the nitrocellulose matrix of the test strip by capillary action. HbA1c from the blood competes with fluorescence-labeled HbA1c peptides for binding sites on HbA1c antibodies fixed on the nitrocellulose matrix. As a result, the higher concentration of HbA1c produces a lower fluorescence signal from HbA1c-peptides. The signal is interpreted and the result displayed on *i*-CHROMA reader in units of percentage.

2.2.5 Estimation of total cholesterol:

Test principle:

Determination of cholesterol after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic of peroxidise (Trinder's reaction).

$$\text{Cholesterol Ester} + H_2O \xrightarrow{\text{esterase}} \text{Cholesterol} + \text{fatty acid}$$

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

$$2H_2O_2 + \text{phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonimine} + 4H_2O$$

The intensity of the pink/red color is proportional to the Cholesterol concentration in the sample. It is determined by measuring the increase the absorbance at 512nm.

Calculation:

ChemWell analyzer automatically calculate the analyte concentration of each sample.

2.2.6 Estimation of triglycerides:

Test principle:

Determination of triglycerides was carried out using the enzymatic splitting with lipoprotein lipase. Indicator is quinoneimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase.

triglycerides $\xrightarrow{\text{Lipoprotein lipase}}$ glycerol + fatty acid

glycerol + ATp $\xrightarrow{\text{Glycerol}}$ glycerol-3-phosphate + ADP

glycerol-3-phosphate +O2 $\xrightarrow{\text{G-3-phosphate oxidase}}$ dihydroxyacetone phosphate + H2O2

2H2O2 + Aminoantipyrine + 4-chlorophenol $\xrightarrow{\text{peroxidase}}$ Quinoneimine +HCL+4H2O

The absorbance of colored complex at 546nm which is proportional to triglycerides concentration.

Calculation:

ChemWell analyzer automatically calculate the analyte concentration of each sample.

2.2.7 Estimation of high density lipoprotein cholesterol(HDL-C)

Test principle

Lipoprotein are particles comprising of a mixture of lipids, phospholipids and apoproteins. There are four distinct groups of lipoproteins: chylomicron, very low density lipoproteins(VLDL), low density lipoproteins(LDL) and high density lipoproteins(HDL).

In the Dialab HDL cholesterol-AUTO Reagent, separation of the lipoprotein fractions is achieved by adding antibodies, which absorb to the surface of the chylomicrons,

VLDL and LDL. In a second step, adding detergent breaks up the HDL lipoproteins, and therefore making HDL cholesterol available for quantification, using an enzymatic system.

LDL, VLDL and Chylomicrons $\xrightarrow{\text{Anti-human β lipoprotein antibodies}}$ Antigen-Antibodies complexes + HDL

HDL-cholesterol + H2O +O2 $\xrightarrow{\text{Esterase α oxidase}}$ cholesten-3-on +fatty acid + H2O2

H2O2 + 4-aminoantipyrine + N-Ethyl-N-(2-Hydroxy-3-sulfopropyl)-3,5-Dimethoxy 4-flouroanilline,

sodium salt $\xrightarrow{\text{peroxidase}}$ blue colored complex + H2O

The color intensity of the dye is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583nm.

Calculation:

ChemWell analyzer automatically calculate the analyte concentration of each sample.

2.2.8 Estimation of low density lipoprotein- cholesterol(LDL-C)

ChemWell analyzer automatically calculate the LDL-cholesterol concentration of each sample using the Freidwald's formula.

LDL = (TCHOL – TAG/5)–HDL-C

2.3 Extraction of DNA from whole blood

Extraction of DNA was carried out using QIAamp DNA Blood Mini Kit (Qiagen, Germany):

2.3.1 Table 1.2 (Kit contents):

Number of preparation	250
QIAamp spin columns	250
Collection tubes(2ml)	750

Buffer AL	54 ml
Buffer AW1(concentrate)	95 ml
Buffer AW2(concentrate)	66 ml
Buffer AE	60 ml
GIAGEN protease	1 vial
Protease solvent	5.5ml

2.3.2 Preparation of reagents:

1. QIAGEN protease stock solution(stored at 2-8C or at -20C)
   Five and a half(5.5 ml) ml of protease solvent were pippeted into the vial containing lyophilized QIAGEN protease, as indicated on the label.
2. Buffer AL(stored at room temperature, 15-25C) was mixed it thoroughly by shaking before use.
3. Buffer AW1 was supplied as a concentrate. Before using the first time, the appropriate amount of ethanol(96-100%) was added to AW1 concentrate as indicated on the bottle and in table 4. Buffer AW1 is stable for 1 year when stored closed at room temperature(15-25C).
   Table(2.2)Preparation of buffer AW1

Kit size	AW1concentrate(ml)	Ethanol(ml)	Final volume(ml)
50	19	25	44
250	95	125	220

4. Buffer AW2 was supplied as a concentrate. Before using for the first time, the appropriate amount of ethanol(96-100%) was added to buffer AW2 concentrate as indicated on the bottle and in table 5. Buffer AW2 is stable for 1 year when stored closed at room temperature(15-25C)

   Table(3.2)Preparation of buffer AW2

Kit size	AW2 concentrate(ml)	Ethanol(ml)	Final volume(ml)
50	13	30	43
250	66	160	226

2.3.3 Blood spin protocol:

1- Twenty µl (20µl) of QIAGEN protease were pipetted into the bottom of a 1.5 ml microfuge tube.
2- Two hundred µl (200µl) of sample were added to the microfuge tube.
3- Two hundred µl (200µl) of buffer AL were added to the sample, and mixed by pulse-vortexing for 15 sec.
4- The mix was incubated at 56° C for 10 min.
5- The mix was then centrifuged briefly to remove drops from the inside of the lid.
6- Two hundred µl (200µl) of 96-100% ethanol were added to the sample, and the contents were mixed again by pulse-vortexing for 15 sec. The mix was centrifuged briefly following the mixing step to remove drops from the inside to the lid.
7- The mixture from step 6 was dispensed carefully into the QIAamp spin column (in a 2ml collection tube) without wetting the rim, The cap was closed, and the column in the tube was centrifuged at 6000g for 1 min. The QIAamp spin column was replaced in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
8- The QIAamp spin column was opened carefully and 500µl buffer AW1 were added without wetting the rim. The cap was closed and the column in the tube was centrifuged at 6000g for 1 min. The QIAamp spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded.
9- The QIAamp spin column was opened and 500µl buffer AW2 were added without wetting the rim. The cap was closed and the column was centrifuged at full speed for 5 min. The QIAamp spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded.
10- To eliminate any chance of possible buffer AW2 carryover, the QIAamp spin column was placed in a clean 1.5ml microfuge and centrifuged at full speed for 1 min.
11- The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp spin column was opened and added 200µl buffer AE, incubated at room

temperature(15-25C) for 1 min, and then centrifuged at 6000xg(8000rpm) for 1min.

For long-term storage, DNA was eluted in buffer AE and stored at -20° C

2.3.4 Determine the concentration, yield and purity of a DNA sample

DNA concentration and purity was measured by two methods: the first method that each DNA sample was estimated by measuring the absorbance at 260nm, adjusting the A260 measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that A260 of 1.0 =50µg/ml pure dsDNA

Concentration(µg/ml) = (A260reading – A320reading) × dilution factor × 50µg/ml

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield(µg) = DNA concentration × total sample volume(ml).

The most common purity calculation was used : the ratio of the absorbance at 260 nm divided by the ratio at 280 nm. Good-quality DNA should have an A260/A280 ratio of 1.7-2.0

The second method , DNA was loaded on 1% agarose gel and electrophoresis as in figure(1.2)

2.4 Amplification of extracted DNA was by using polymerase chain reaction(PCR) gradient thermal cycler TC-5000.

2.4.1 Ethanol precipitation of DNA

Reagent needed

- 5M ammonium acetate
- DNA
- 100% Ethanol

Protocol

1- The volume of the DNA sample was measured.

2- 1/2 volume of ammonium acetate were added to the sample.

3- The contents mixed well.

4- 2 to 2.5 volumes of cold 100% ethanol(calculated after salt addition)were added to the mix.

5- The contents mixed well again.

6- The tubes were placed on ice or at -20 degrees C for > 20 minutes.

7- The tubes were spun at a maximum speed in a microfuge for 10-15 min.

8-Supernatant was decanted carefully

9- One ml 70% ethanol was added, mixed and spun briefly. supernatant was decanted carefully

10- Pellet was air dried.

11- pellet was resuspended in the appropriate volume of TE or water.

2.4.2 Resuspending PCR Primers

The sequences of the primers of the Homo sapiens GSTP1(exon 5) were obtained from the work published by (Amer et al., 2012). They were as follow:

Forward primer(A) of GSTP1(exon 5) is (5'-ACC CCA GGG CTC TAT GGG AA-3')

Reverse primer(B) of GSTP1(exon 5) is (5'-TGA GGG CAC AAG AAG CCC CT-3')

Materials

- RNase free water
- Primers(dry)
- Sterile Microcentrifuge Tubes

Method

Primers supplied as lyophilized were used to create a master of 100x stock for each primer, which was then diluted to a 10x of working stock.

This reduces the number of freeze/thaw cycles that master primer stock goes through and reduces the chances of contaminating the primary source for the primer.

Master stock,100µM

The amount of H2O to add to the lyophilized primer was determined by simply multiplying number of nmole of the primer in the tube by10 and that makes the amount of add to make a 100µM primer stock.

The number of nmole of Primer A(forward) was 22.5nm, so the amount of H2O added = 22.5 × 10 = 225µl.

The amount of nmole of Primer B(reverse) was 33.4nm, so the amount of H2O added= 33.4 × 10 = 334µl.

Master stock primers newly suspended in H2O should be allowed to sit at room temperature for 10 minutes before they were used for working stock dilutions. mixed well before making working stock dilutions.

Working stock, 10µM

The primer master stock in a sterile microcentrifuge tube were diluted 1:10 with DNase free water.

5µl of stock solution were required 45µl of RNase water to make working primer reagent.

2.4.3 Taq DNA Polymerase and Taq PCR Core Kit

Table(4.2) Reaction set up using Taq DNA polymerase.

Component	Volume/reaction	Final concentration
Reaction mix 10xPCR buffer *	5µl	1X

or **optional**: 10x CoralLoad PCR buffer*		
dNTP mix(10mM of each)	1µl	200µM of each dNTP
Primer A	1.5µl	0.1–0.5µM
Primer B	1.5µl	0.1–0.5µM
Taq DNA polymerase	0.25µl	2.5units/reaction
RNase free water	27.75µl	–
Optional: 5x Q-solution†	10µl	1x
Template DNA(added at step4)	3µl	≤1µg/reaction
Total reaction volume	50µl	

*Contains 15mM MgCl2.

† For template with GC-rich regions or complex secondary structure

1-The 10x CoralLoad PCR Buffer or 10x PCR Buffer, dNTP mix, primer solutions, and 25Mm $MgCl_2$ were thawed at room temperature or on ice. Thawing is followed by thorough mixing to avoid localized differences in salt concentration.

2-The reaction mix was prepared according to table (4.2). The used reaction mix typically contained all of the components needed for PCR. A volume of reaction mix 10% greater than that required for the total number of PCR assays to be performed was prepared.

3-The reaction mix was mixed gently but thoroughly by pipetting up and down for a few times. An appropriate volume was dispensed into the PCR tubes.

4-DNase free water was added to one PCR tube, and the DNA template to the rest PCR tubes containing the reaction mix.

5-The thermal cycler was programmed according to the manufacturer's instructions. A typical PCR cycling program was used and outlined in table (5.2)

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Table (5.2) optimized cycling conditions

Step	time	temperature	comment
Initial denaturation	3 min	94°C	
3-step cycling Denaturation	0.5-1 min	94°C	Approximately 5°C below Tm of primers
Annealing	0.5-1 min	50-68°C	For PCR products longer than 1 kb, use an extension time of approximately 1 min per kb DNA
Extension	1 min	72°C	
Numbers of cycles	35		
Final extension	10 min	72°C	

6-When using CoralLoad PCR Buffer, the PCR products can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel-tracking dyes.

2.5 Recommended protocol for digestion of PCR products directly after amplification

Table (6.2)protocol for digestion of PCR products

1-Added	
PCR reaction mixture	10µL(˷0.1-0.5µg of DNA)
Nuclease-free water	18µL
10X buffer Tango	2µL
ALW26I	1-2µLL
2- Mix gently and spin down for a few seconds.	
3-Incubate at 37ºC for 1-16 hours.	

2.6 Protocol of loading PCR products before and after digestion on agarose gel

1-Agarose in the quantity of 1.25g were weighed and dissolved in 50µL of 1x TAE buffer by heating the solution on hot plate until the agarose dissolved completely.

TAE: To prepare 10x stock solution in 1liter of H_2O

48.4g Tris base [tris (hydroxymethyl) aminomethane]

11.4ml glacial acetic acid (17.4M)

20ml of 0.5M EDTA or 3.7g EDTA, disodium salt

All were dissolved in 800ml deionized water and made up to 1 liter, stored at room temperature and the solution was diluted to 1x prior to use [100ml (10x stock) up to 1 liter deionized water].

2-Insulated gloves were used to transfer the flask until the gel has cooled at 55ºC .

3-The warm agarose solution was poured into the BioRad mold (17x17) using an appropriate comb.

4-The gel was left to polymerize completely (20-45 minutes at room temperature), then an appropriate electrophoresis buffer was used to cover the gel to a depth of approx. 1mm.

5-The sample was mixed with loading dye with a ratio of 1:10.

6-The sample mixture was loaded into the slots of the submerged gel. Size standards was loaded into slots on both the right and left sides of the gel.

7-The lid of the gel tank was closed and the electrical leads were attached so that the DNA migrated towards the positive anode (red lead). A voltage of 1-5 V/cm was applied. Bubbles generated at the anode and the cathode indicating running of current, and within a few minutes the bromophenol blue started to migrate from the wells into the body of the gel. The current was left to run until the bromophenol blue and xylene cyanol FF had migrated to the last third of the gel.

8- When the DNA samples or dyes have migrated for a sufficient distance through the gel, the electric current was stopped and the leads removed and from the gel tank.

9-The gel was stained by immersing it in electrophoresis buffer containing ethidium bromide (0.5µg/ml) for 20-45 minutes at room temperature. It was then destained by immersing it in electrophoresis buffer.

2.7 Detection of DNA in agarose gels

Nucleic acids were detected by visualizing under 300-nm UV light using geldoc XR BioRad camera for getting images of ethidium bromide stained gel as in figure(9.3)

2.8 Detection of GSTP1Ile105Val by high resolution melting(HRM) analysis

Principle:-

High resolution melting analysis is an innovative technique that is based on analysis of DNA melting. HRM characterizes DNA samples according to their association behavior as they transition from double-stranded DNA(dsDNA) to single-stranded DNA(ssDNA) with increasing temperature.

Before performing HRM analysis, the target sequence were amplified to a high copy number in the presence of the dsDNA-binding fluorescent dye, Evagreen. The dye does not interact ssDNA but actively binds dsDNA and fluoresces brightly when bound. Change in the fluorescence was used to measure the increase in DNA concentration during PCR and then to directly measure thermally –induced DNA melting by HRM.

To perform high resolution melting analysis, the temperature was increased from a lower to high temperature. The fluorescence of EvaGreen was measured continuously as the temperature was increased and was plotted against the temperature. EvaGreen is fluoresces as long as it is bound to dsDNA. Due to the amplification procedure before the HRM analysis, fluorescence is high at the beginning of the HRM analysis. Upon melting of dsDNA, EvaGreen is released and the fluorescence is reduced to a background level.

Procedure:-

- Twice the concentrations of HRM PCR master mix, primer solution, RNase-free water, template DNAs were thawed.
- A reaction mix was prepared according to table 7.2

Table (7.2) preparation of reaction mixture

Component	Volume per 25µl reaction*	Volume per 10µl	Final concentration
2xHRM PCR Master Mix	12.5µl	5.0µl	1x
10µM primer mix†	1.75µl	0.7µl	0.7µM forward primer, 0.7µM reverse primer
RNase-free water	Variable	variable	-
Template DNA	Variable(equal volume for all reaction)	Variable(equal volume for all reaction)	Eukaryotic:1-50ng DNA/reaction Microbial: 1-50ng DNA/reaction
Total volume per reaction	25µl*	10µl	-

*If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.
† A 10µM primer mix consists of 10µM forward primer and 10µM of reverse primer.
It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.

- The reaction contents were mixed thoroughly, and the appropriate volumes were dispensed into the PCR tubes or the wells of a PCR plate.
- An equal amounts and volumes of template DNA (1-50ng genomic DNA or 1-50ng microbial DNA, same amount for each sample) were added to the individual PCR tube or well and mixed thoroughly.
- The real- time cycler was programmed according to table 8.2

Table(8.2) real-time cycler program

Step	Time	Temp.	Additional comments
Initial PCR activation step	5min.	95ºC	Hotstar Taq *plus* DNA polymerase is activated by this step
3-step cycling Denaturation	10s	95ºC	

Annealing	30s	55ºC	
Extension	10s	72ºC	Activate ″single″ fluorescence data acquisition. Suitable for PCR products up to 350bp. For PCR products>350bp, use 1s extension time per 25 bp of PCR product length.
Number of cycles	40 45		10-50ng template or microbial DNA 1-9ng template or microbial DNA
HRM	2s	65-95ºC 0.1C increments	Fluorescence data acquisition

- The PCR tubes or plate were placed in the real- time cycler, and the PCR cycling program was started, followed by HRM analysis.
- Data analysis were performed as in figure(11.3).

3 -Results

This current study is an attempt to elucidate the role of glutathione-s-transferase p1genetic variant in the type 2 diabetes mellitus and its effect on glycemic parameters using different molecular techniques. The study involved 67 patients and 26 healthy control.

The mean age and standard deviation(SD) of the patients with type2 DM selected for this study was 55.60±9.983, and the female: male ratio was 5:6. The age range was(35-65) years. The mean age and SD of healthy control subjects was 50.00±8.080, and the female: male ratio was 5:8. The age range was(37-63) years.

3.1 Body mass index(BMI)

The mean and standard deviation of the body mass index of the patients with type2 DM and healthy control subjects selected for this study was 29.7821±1.71623 and 24.3885±0.59553 respectively. BMI was significantly higher in diabetic group when compared to control group($p<0.05$). (Table 1.3, Figure 1.3).

3.2 Blood pressure(BP)

The mean and standard deviation of systolic blood pressure of patients with type2DM and healthy control subjects selected for this study was 117.18±9.210 and 113.35±5.455 respectively, the mean and standard deviation of diastolic blood pressure of patients and healthy control subjects was 82.46±7.384 and 76.08±2.682 respectively. Systolic and diastolic blood pressure measurements showed significant differences comparing diabetic patients to the control group($p<0.05$). (Table 2.3, Figure 2.3).

3.3 fasting blood glucose(FBG)

the mean and standard deviation of fasting blood glucose of patients and healthy control subjects was 163.37±63.934 and 89.69±6.781 respectively. Diabetic patients had significantly higher fast blood glucose than healthy control group ($p<0.05$). (Table 3.3, Figure3.3).

3.4 Glycosylated hemoglobin(HbA1c)

The mean and standard deviation of glycosylated hemoglobin of patients and healthy control subjects was 7.816±1.7807 and 5.100±0.4147 respectively. Diabetic patients

had significantly increase in HbA1c when compared to healthy subjects ($p<0.05$).(Table 4.3 Figure 4.3).

3.5 Serum total cholesterol

The mean and standard deviation of total cholesterol in patient and healthy control subjects was 182.54±34.584 and 168.09±24.478 respectively. The mean level of the serum total cholesterol in patients with type2 DM was higher than that of the control subjects and the difference was statistically significant ($p<0.05$).(Table 5.3, Figure 5.3).

3.6 High density lipoprotein-cholesterol(HDL-C)

The mean and standard deviation of the high density lipoprotein of patients and healthy control subjects was 46.343±9.2007 and 67.131±10.428 respectively. Patients with type2 DM had significantly lower HDL-C than normal control subjects ($p>0.05$). (Table 6.3, Figure 6.3).

3.7 low density lipoprotein-cholesterol(LDL-C)

The mean and standard deviation of low density lipoprotein of patients and healthy control subjects was 106.985±34.4616 and 92.981±19.4410 respectively. LDL-C was significantly higher in diabetic patients when compared to the normal control($p<0.05$). (Table 7.3, Figure 7.3).

3.8 Serum triacylglycerol(TAG)

The mean and standard deviation of serum triacylglycerol of patients and healthy control subjects was 144.31±69.231 and 119.75±43.195 respectively. Mean TAG levels were higher in the diabetic groups as compared to the control group and the difference was statistically significant ($p<0.05$).(Table 8.3, figure 8.3)

3.9 Size of DNA bands after extraction from blood

Following the extraction of DNA from blood samples were blotted on 1% agarose gel to detect the desired DNA extraction as in figure(9.3).

3.10 Running of DNA sample after digestion on 2.5% agarose gel electrophoresis

Following PCR of the GSTP1 gene by using the forward and reverse primers and after digest PCR product with restriction enzyme ALW261 overnight DNA samples were plotted on 2.5% agarose gel as in figure(10.3).

After running 25 samples of healthy control subjects including(14 male and 11female) and 18 samples of type2 DM including(8 male and 10 female), the G allele(val) was more prevalent among healthy control subjects than among patients. We found that 6 of control subjects (including 2 female and 4 male) and 4 of patients(including 3 female and 1 male) were heterozygous for the G allele(Ile/val) which is shown on agarose gel as two bands, one band has size of 176bp for normal locus on allele and another band has size 85bp+91bp (which is shown as one band due to very little small differences in base pairs between them) for mutant allele on another allele as in figure(10.3).

G allele homozygosity(val/val) detected in 3 sample of control subjects (including 2 female and 1 male), whereas in patients 1 male was homozygosity for G allele(val/val) which is shown on agarose gel as one band has size 85bp+91bp for mutant locus on both alleles as in figure(10.3).

In detection of GSTP1Ile105Val by high resolution melting analysis we found 2 samples of healthy control subjects and 2 samples of type 2 DM patients were heterozygous for allele G(which their melting curve are shown the lowest curves compared to the ones with normal and homozygous samples), while 1 sample of control and 1 sample of patient were homozygous for allele G. 13 samples of type 2 DM patient and 3 samples of control subjects were homozygous for wild allele A(which their melting curve are shown the highest curves) as in figure(11.3).

Table 1.3: mean± SD of body mass index(BMI) in diabetic and healthy groups.

Groups	Patients N= 67	Controls N= 26	P value
BMI(kg/m2)	29.7821±1.71623	24.3885±0.59553	0.000

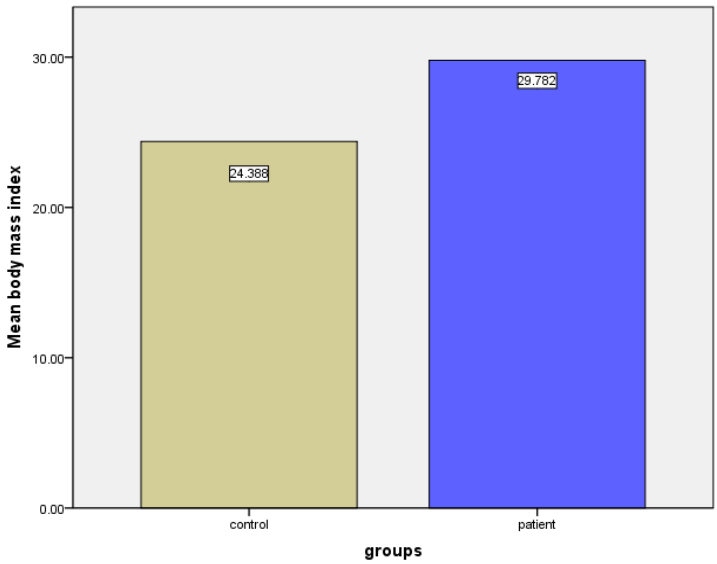

Figure 1.3:mean of body mass index (BMI) in diabetic and healthy groups

Table 2.3 mean± SD of blood pressure in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
Diastolic mmHg	82.46±7.384	76.08±2.682	0.000
Systolic mmHg	117.18±9.210	113.35±5.455	0.010

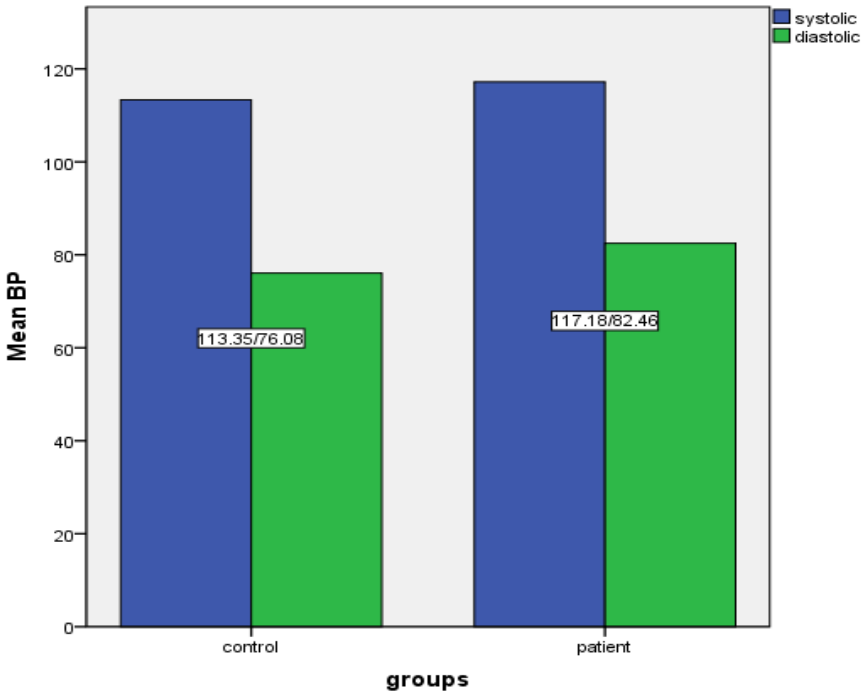

Figure 2.3: mean of blood pressure in diabetic and healthy groups.

Table 3.3: mean± SD of fasting blood glucose in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
Fasting blood glucose	163.37±63.934	89.69±6.781	0.000

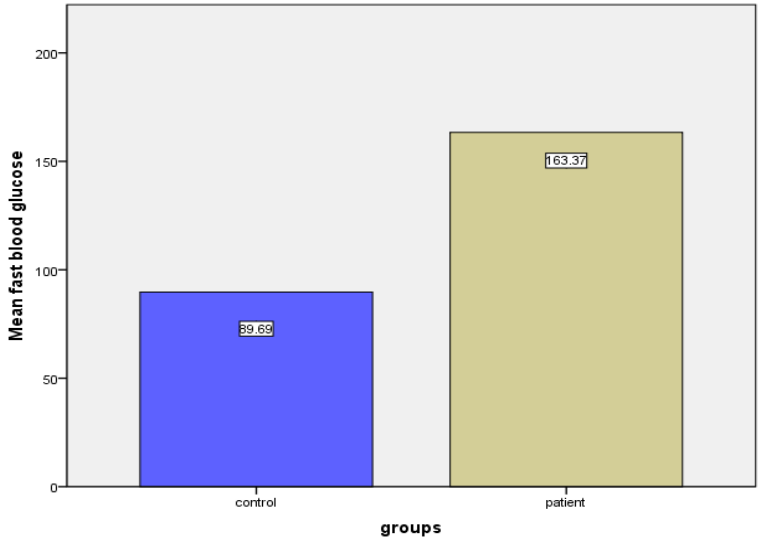

Figure 3.3:mean of fasting blood glucose in diabetic and healthy groups.

Table 4.3: mean± SD of glycosylated hemoglobin in diabetic and healthy groups.

Groups	Patients N=67	Control N=26	P value
Glycosylated hemoglobin	7.816±1.7807	5.100±0.4147	0.000

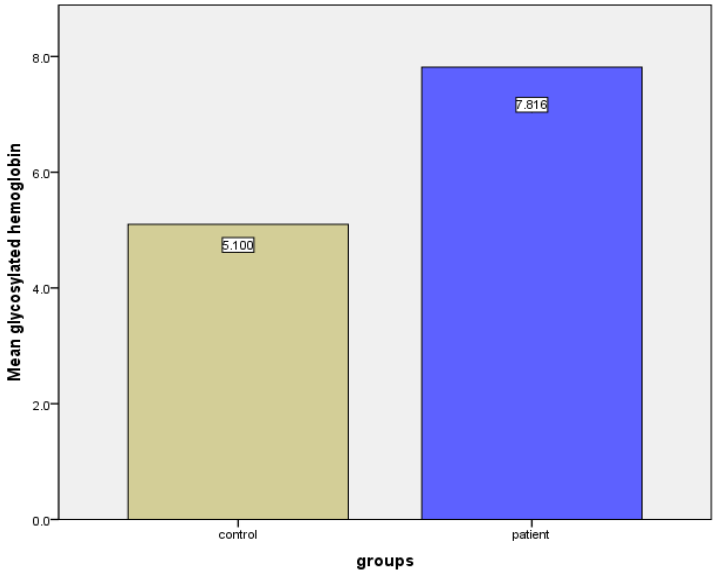

Figure 4.3:mean of glycosylated hemoglobin in diabetic and healthy groups.

Table 5.3: mean±SD of serum total cholesterol in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
Total cholesterol	182.54±34.584	168.09±24.478	0.027

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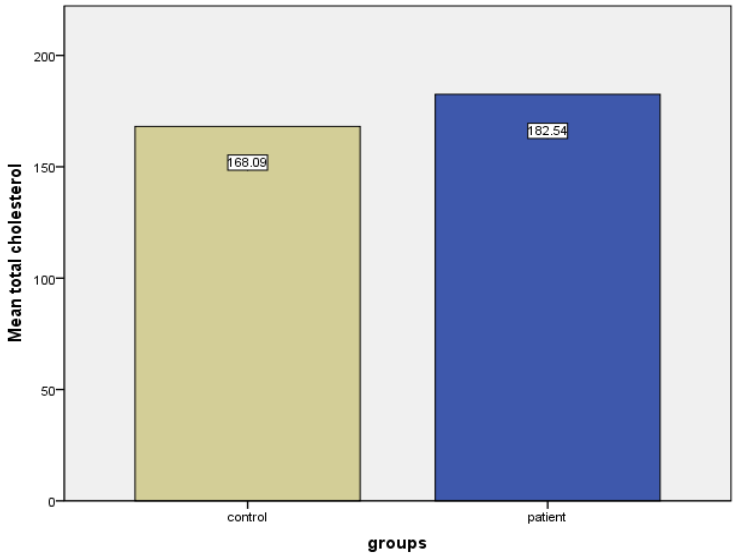


Figure 5.3: mean of serum total cholesterol in diabetic and healthy groups.

Table 6.3: mean± SD of HDL-C in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
HDL	46.343±9.2007	67.131±10.428	0.000

Figure 6.3:mean of HDL-C in diabetic and healthy groups

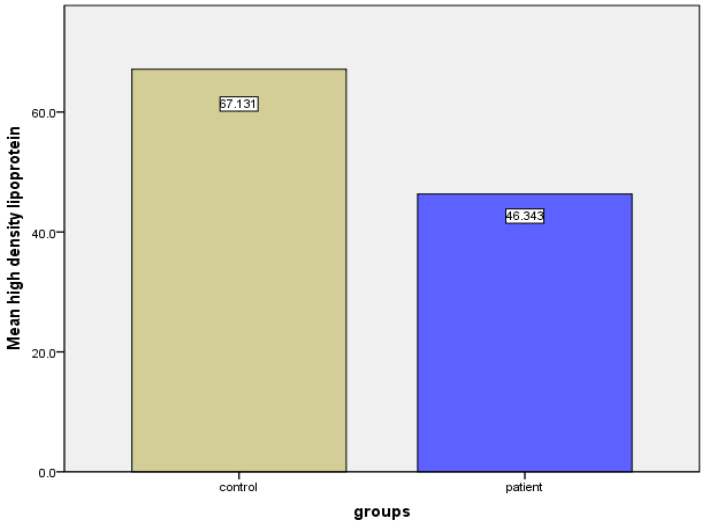


Table 7.3: mean±SD of LDL-C in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
LDL-c	106.985±34.4616	92.981±19.4410	0.016

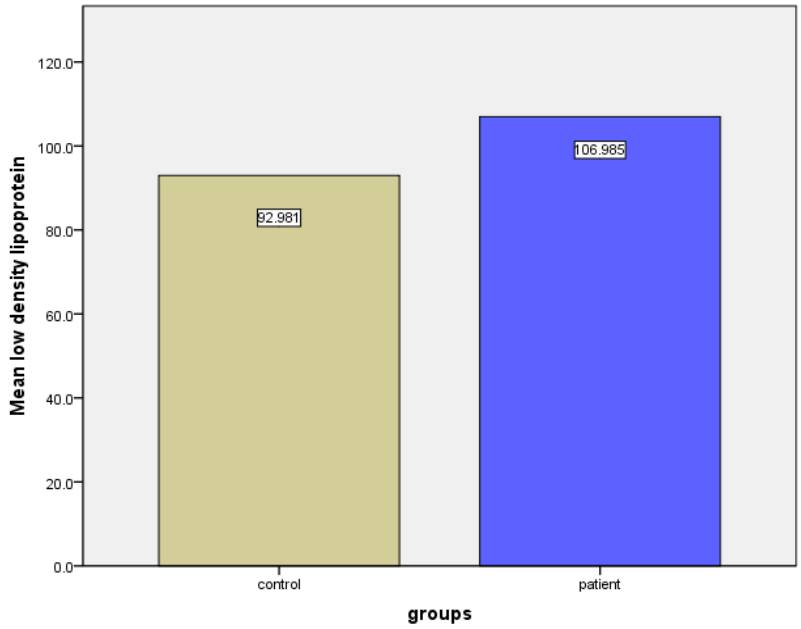


Figure 7.3:mean of LDL-C in diabetic and healthy groups.

Table 8.3: mean± SD of serum triacylglycerol in diabetic and healthy groups.

Groups	Patients N=67	Controls N=26	P value
TAG	144.31±69.231	119.75±43.195	0.040

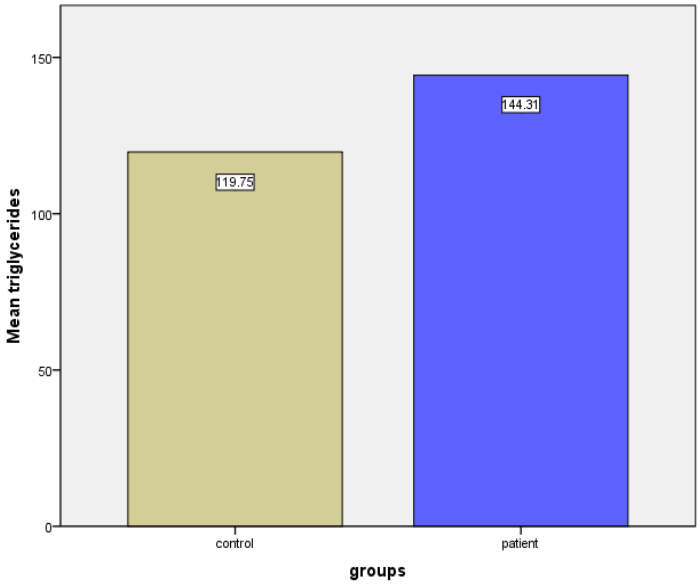
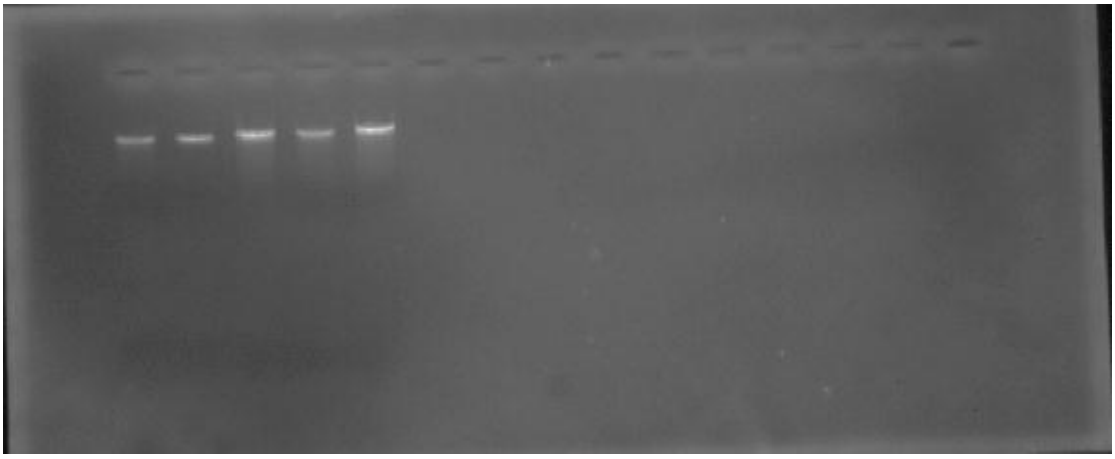
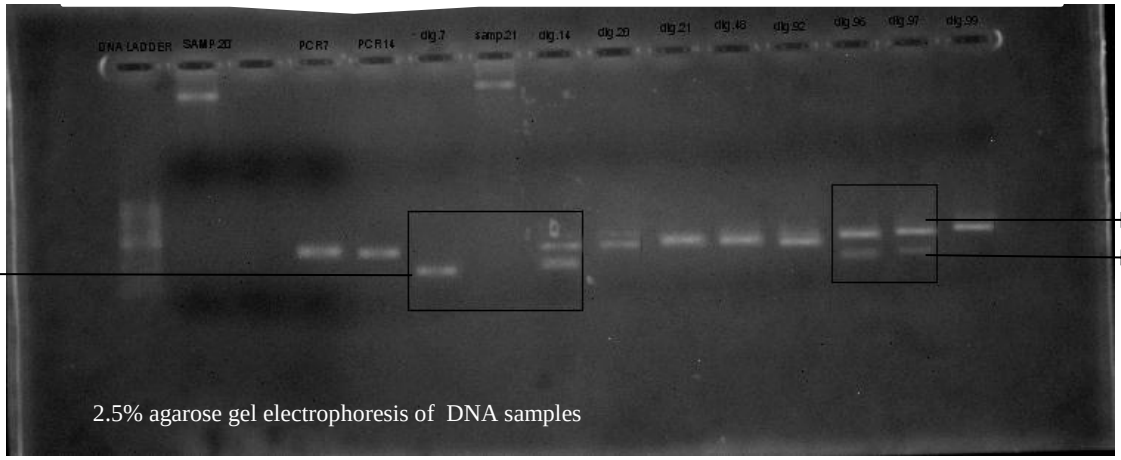


Figure 8.3: mean of serum triacylglycerol in diabetic and healthy groups.



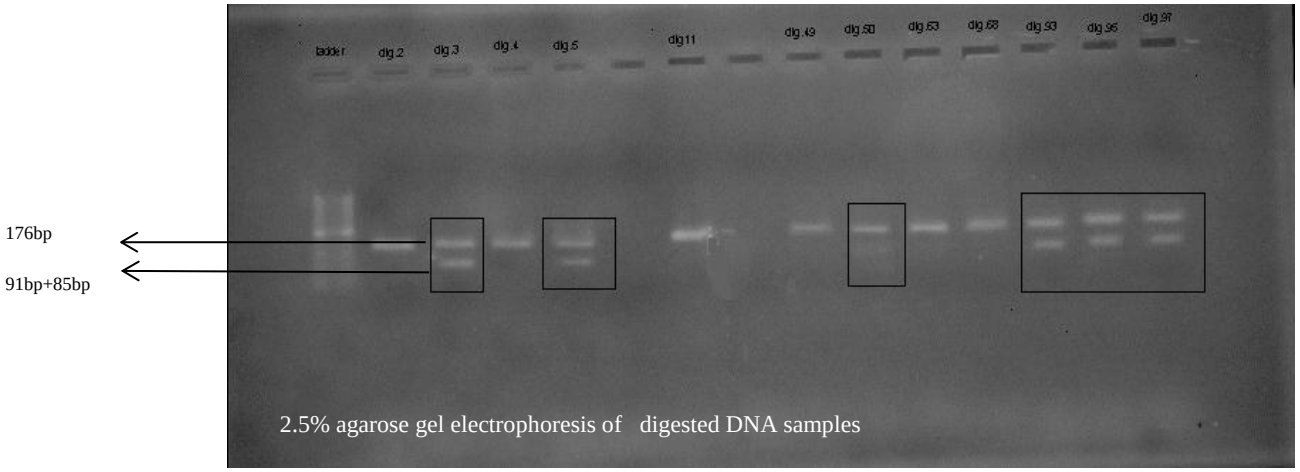
Figure(9.3) Running of some DNA samples after extraction on 1% agarose gel after extraction

M P1 P2 P3 N1 P4 P5 P6 P7 P8 N2 N3 N4 N5



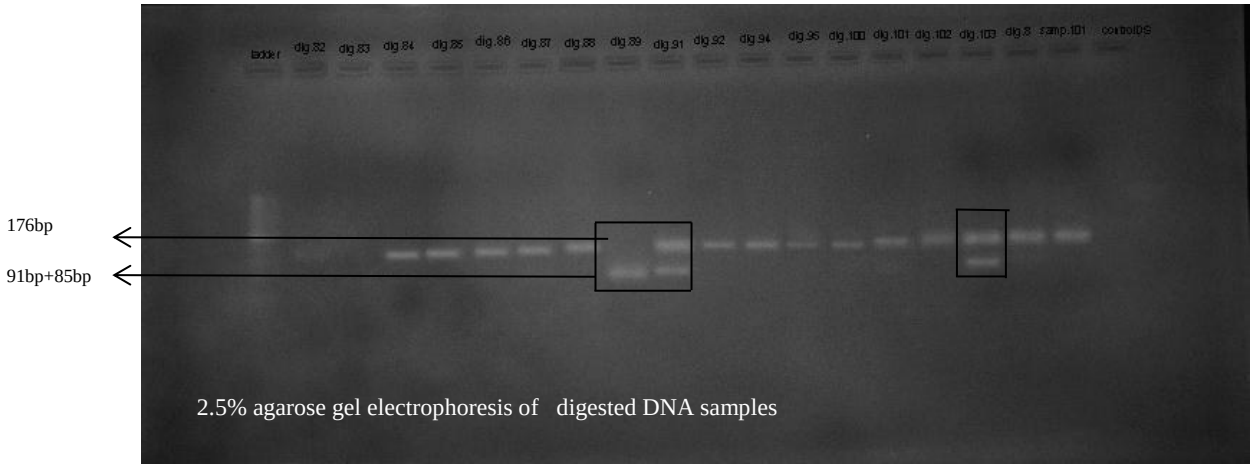
P1and P4 showing DNA sample after extraction, P2and P3 showing PCR product of sample, P5,N3 and N4 showing PCR product of samples after digestion overnight with ALW261 and samples are heterozygous carrying A allele(176bp) and G allele which cuts by ALW261 into two bands one with 91 bp and other with 85bp, P6, P7, P8,N2 and N5 showing PCR products of samples after digestion overnight with ALW261 and samples are homozygous carrying two normal A alleles, while N1 showing PCR product of sample after digestion overnight with ALW261 and sample is homozygous carrying two polymorphic G allele.

M P1 P2 P3 P4 N1 P5 P6 P7 P8 N2 N3 N4



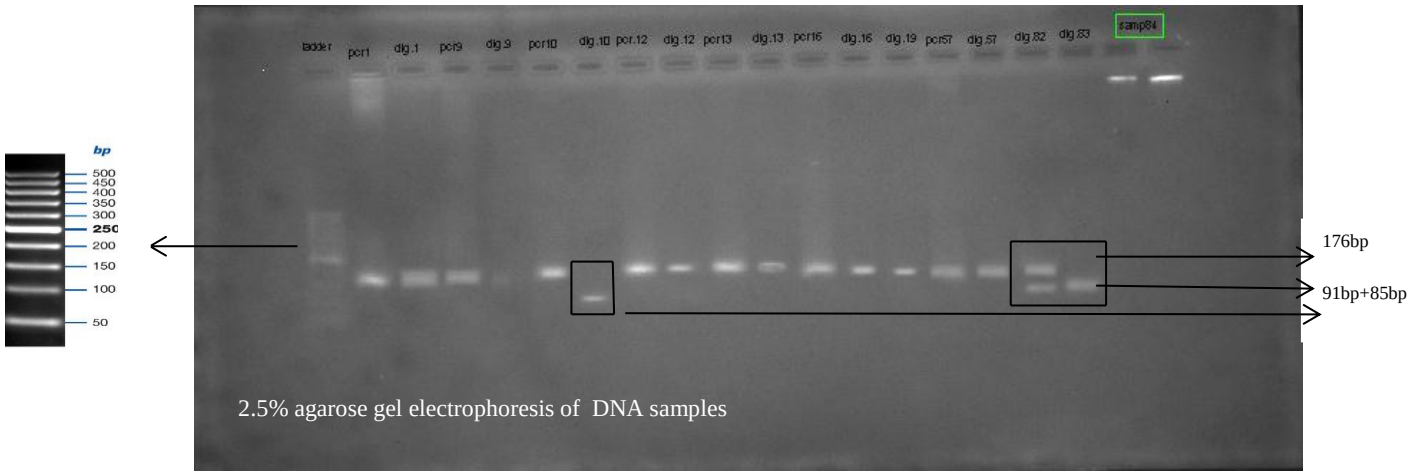
P2,P4,P6, N2,N3 and N4 showing PCR product of samples after digestion overnight with ALW261 and samples are heterozygous carrying A allele(176bp) and G allele which cuts by ALW261 into two bands one with 91 bp and other with 85bp. P1, P3, P5,P7, P8 and N1 showing PCR products of samples after digestion overnight with ALW261 and samples are homozygous carrying two normal A alleles.

M N1 N2 N3 N4 N5 N6 N7 N8 N9 N10 N11N12 N13 N14 N15N16N17N18 C

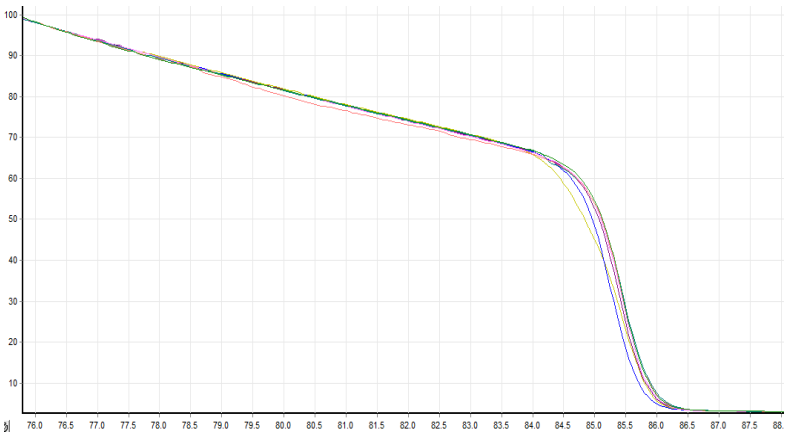


N9 and N16 showing PCR product of samples after digestion overnight with ALW261 and samples are heterozygous carrying A allele(176bp) and G allele which cuts by ALW261 into two bands one with 91 bp and other with 85bp. N1,N2,N3,N4,N5,N6,N7,N10,N11,N12,N13,N14,N15,N17 and N18 showing PCR products of samples after digestion overnight with ALW261 and samples are homozygous carrying two normal A alleles, while N8 showing PCR product of sample after digestion overnight with ALW261 and sample is homozygous carrying two polymorphic G allele.

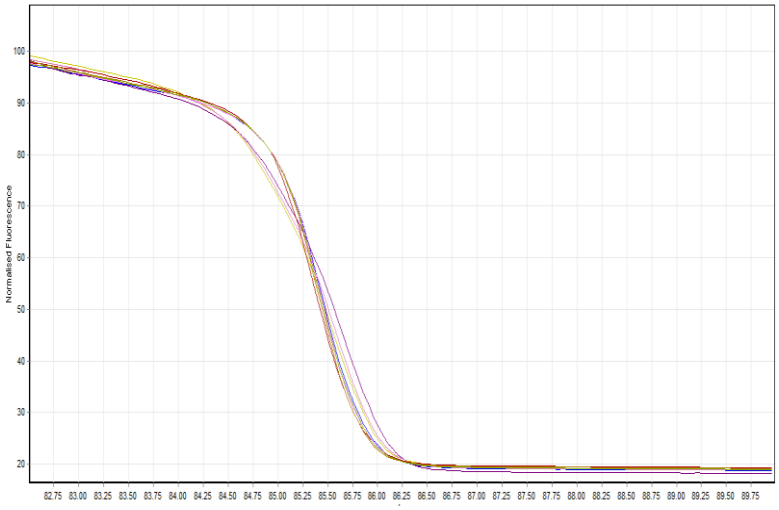
M P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11P12P13 P14 P15 N1 N2 N3



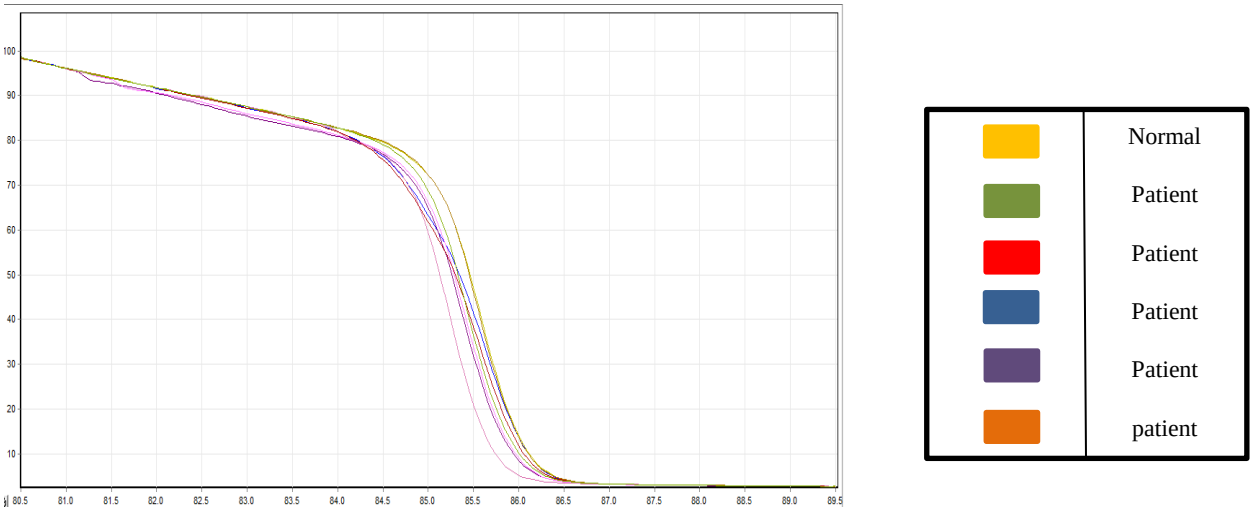
Figure(10.3)Running of some DNA samples on 2.5% agarose gel electrophoresis after digestion with ALW261. (M = marker N = normal P = patient C = control). P1,P3,P5,P7,P9,P11 and P14 showing PCR product of samples and samples are homozygous carrying two normal A alleles. N1 showing PCR product of sample after digestion overnight with ALW261 and sample is heterozygous carrying A allele(176bp) and G allele which cuts by ALW261 into two bands one with 91 bp and other with 85bp. P6 and N2 showing PCR products of samples after digestion overnight with ALW261 and samples are homozygous carrying two normal A alleles, while N3 showing sample after extraction.



	Normal
	Normal
	Patient
	Patient
	Normal



	Patient
	Normal
	patient
	Normal
	patient



Figure(11.3)analysis of some DNA samples by using HRM Rotor-Gene Q series software, showing the curves with the highest melting point like yellow and brown curves in the last figure refer to the samples carrying two G alleles, while the curves with the lowest melting point like yellow and violet curves in the second figure refer to samples carrying A&G alleles and the curves lie in the middle the curves with the highest and lowest refer to sample carrying two A alleles.

4 –Discussion

In our present case-control study of 67 type 2 diabetic patients and 26 normal control Subjects. All required glycemic and lipid profile parameters for both groups including (FBG, HbA1c, LDL, HDL,VLDL, TAG and total cholesterol) in addition to BMI and systolic/ diastolic blood pressure were measured and they were significantly different in diabetes compared to controls. GSTp1Ile105Val is a genetic variant of normal GSTP1 isoenzyme which has amino acid valine instead isoleusine at codon 105 of exon 5 with lower activity toward ROS that increase the complications of diabetes. In our study, DNA was genotyped for GSTP1–313 A> G (Ile105Val) polymorphism using the PCR–restriction fragment length polymorphism technique to show if there is any association of type 2 diabetes with GSTP1Ile105Val.

Type 2 diabetes mellitus, obesity and the metabolic syndrome are strongly correlated with increased skeletal muscle content of reactive oxygen species(ROS) (Abdul-Ghani et al., 2008, Bonnard et al., 2008, Kumashiro et al., 2008). When ROS are moderately produced, they are involved in important physiological processes that lead to desired cellular responses. However, high ROS production is negatively associated with different biological signaling pathways (Rains and Jain, 2011). ROS can react with multiple cellular components, such as proteins, lipids and nucleic acids generating reversible or irreversible oxidative modifications. Pathophysiological processes mediated by ROS are more likely to induce irreversible modifications in cellular components, a reasonable definition of the term oxidative stress (Bashan et al., 2009)

Control of vascular tone, cell adhesion, immune responses, and growth factors and hormone action are examples of ROS participation in normal physiology (Gutterman et al., 2005, Taddei et al., 2007). Conversely, a negative role of ROS has been implicated in ageing-related diseases, malignant transformation , atherosclerosis, neurodegenerative diseases, obesity, and diabetes (Wei and Lee, 2002, Madamanchi et al., 2005). The family of GST genes plays an important role in protecting cells from oxidative stress. GSTP1 is the most important member of GST family which contributes to the detoxification of electrophilic compounds by glutathione conjugation (Autrup, 2000). Furthermore, GSTP1 is a key substance in the biotransformation and inactivation of certain environmental pollutant. Due to enzymatic activity of GSTP1-313 A> G polymorphism, GSTP1 may confer

susceptibility to several cancers (Qadri et al., 2011, Huang et al., 2013). GSTP1 catalyzes the detoxification of products arising from DNA oxidation (Hayes and Pulford, 1995). A defect in detoxifying reactive oxygen species, which is genetically determined, may influence the development and severity of diabetes mellitus (Yalin et al., 2007). There are many studies dealing have been focused on the GSTP1-313 A> G polymorphism and the risk of various cancers, including lung, breast, colorectal, bladder, pancreatic, thyroid, and prostate cancer (Huang et al.,2013), but only a few studies have addressed the role of GSTP1 gene polymorphism in diabetes. Therefore, the current study was designed to investigate the role of GST-P1(Ile105Val) gene in diabetes.

Studies have been demonstrated that ROS lead to impaired insulin response by inducing IRS serine/threonine phosphorylation, decreasing GLUT4 gene transcription, and decreasing mitochondrial activity (Bloch-Damti and Bashan, 2005, Morino et al., 2005).

Several investigators have determined the clinical or genetic factors associated with T2DM with interests to detoxification agents. As regards GSTM1,T1 and P1 isoenzymes, studies on Egyptian (Amer et al., 2011),Chinese (Wang et al., 2006), and Brazilian(Pinheiro et al., 2013) populations reported a significant association of the null mutation of GSTT1 gene and T2DM, whereas in studies involving Turkish (Gönül et al., 2012) North Indian (Bid et al., 2010),and Southern Iran (Moasser et al., 2012) populations this association was observed between GSTM1 deletion and T2DM. recently, studies conducted on Japanese (Hori et al., 2007) and south Indian population (Ramprasath et al., 2011) as well as another meta-analysis study involving Asian, European and African diabetic population (Tang et al., 2013) reported the association of both GSTM1 and GSTT1 null genotypes with the risk of developing T2DM. The North Indian (Bid et al.,2010) and another Egyptian study conducted in T2DM were the only ones demonstrating a significant association of the GSTP1 SNP (A313G) with T2DM.

Our results demonstrate that the frequency of the G allele was higher in controls than in patients after detection by running of digested DNA samples on 2.5%agarose gel electrophoresis and by using high resolution melting analysis which depends on melting point of PCR products that during annealing step heteroduplex products

formed in heterozygous samples. The heteroduplex products have the lowest melting point compared to mutated samples which have higher melting point than wild samples. We therefore suggest that the G allele (Val) of GSTP1 Ile105Val does not play an important role in predisposition to T2DM.

There have been controversial results regarding the association between GSTP1 Ile105Val gene polymorphism and diabetes development. We are in agreement with Yalin et al.(Yalin et al.,2007) and Oniki et al.(Oniki et al., 2008), who suggested that GSTP1 Ile105Val polymorphism may not play a significant role in the etiopathogeneses of DM in the Turkish population and Japanese population respectively. In contrast, Ramprasath et al.(Ramprasath et al.,2011) and Bid et al.(Bid et al.,2010) suggested that GSTP1 Ile105Val polymorphism may play a vital role in the development of diabetes mellitus.

These contradictory results might be attributed to different ethnic origins, environmental difference in addition to problems related to research methodologies like the adequacy of sample size and proper diagnostic methods (Elsaid et al., 2015).

The epidemic of T2DM observed in recent years is a clear indication of the importance of environmental factors in diabetes onset, in particular obesity and physical inactivity(Bid et al.,2010). Obesity, mainly when fat is distrusted predominantly at the abdominal level, is the main risk factor for T2DM.

A number of epidemiological studies have tested possible association between polymorphism of the GST isoforms particularly deletion in the GSTM1 and GSTT1 genes(null genotypes) and the GSTP1 A313G SNP with disease risk or therapy outcome in different types of pathologies (Tamer et al., 2004, Moore et al., 2005, Ozerkan et al., 2012).

In our study fasting blood glucose, glycosylated hemoglobin, total cholesterol and triacylglycerol, high density lipoprotein, low density cholesterol, blood pressure and body mass index in healthy control subjects and type 2 DM patients were significantly different (Bloom et al., 1975) BMI was significantly higher in diabetic group than control group, and this finding is consistent with several similar studies (Group, 1998, Daousi et al., 2006, El-Hazmi and Warsy, 1999). It is well documented that obesity increase morbidity and mortality in type 2 diabetic patients and short-term studies

have demonstrated even moderate weight reduction through diet and/or exercise can improve patient hyperglycemia (Maggio and Pi-Sunyer, 2003, Scheen, 2003). However, the few long term cohort studies regarding the effects of weight loss on glycemic control in diabetic patients produced inconsistent results (Aucott et al., 2004, Shoff et al., 1998).

It has been speculated that the inconsistencies may have arisen from the confounding influences of other factors on body weight, such as the disease process itself or the medications used (Heller et al., 1993, Group, 1998, Tseng, 2007)

The present study showed that systolic and diastolic blood pressures were significantly higher in diabetic group when compared to control group. Many studies demonstrated a high prevalence of about 61.2% (Mengesha, 2008).

In comparison with the general population, individuals with type2 diabetes mellitus (DM) have a 2-to 4-fold increased risk factor for cardiovascular disease. Hypertension is an important and modifiable risk factor for cardiovascular disease associated with DM, and the results of many studies suggested that 35% to 75% of the cardiovascular risk in diabetic patients can be attributed to the presence of hypertension (Booth et al., 2006, Lloyd-Jones et al., 2006, Lee et al., 2000)

The present study results showed that FBG was significantly higher in diabetic patients when compared to the controls, and this finding is quite logical, since hyperglycemia is the primary factor that distinguishes diabetic individuals in general population. Furthermore, patients with HbA1c higher than 7.5% had significantly higher levels FBG than patients with HbA1c $\leq$ 7.5%, and FBG showed a direct association with HbA1c. these findings are in agreement with the findings reported by others (VinodMahato et al., 2011, Rosediani et al., 2006, Ramona et al., 2011).

The results of the present study revealed that serum total cholesterol , LDL cholesterol and triacylglycerol were significantly raised, whereas the level of HDL cholesterol was significantly lower in diabetics as compared to control subjects. These observed increases and decreases in serum lipid profile associated with diabetes mellitus are in agreement with the findings of Calismasi, Kandula et al., and Albriki et al.(Çalışması, 2008, Kandula and Shegokarz, 2013, Albrki et al., 2007).

5 – Conclusion

We designed this study to elucidate the role of GSTP1 genetic variant in the type 2 diabetes mellitus in Libyan patients.

This is the first study to determine the association of type 2 diabetes with GSTP1Ile105Val gene polymorphism in the Libyan population.

Our results showed that GSTP1 Ile/Val genotype may not play a significant role in the etiopathogeneses of T2DM.

We acknowledge that the findings presented here are preliminary because of the small number of subjects and that the study requires confirmation in a separate, larger cohort. Additionally, a wide epidemiological study is needed to test the possible association between genotypic and phenotypic effects of other genetic polymorphisms in GST isoenzymes in diabetic patients such as null GSTM and GSTT.

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Appendix I

(Questionnaire)

File NO. _____ Patient Name: _____

Age: …….. . **Gender: M ( ) F ( ) Initial diagnosis:** ……. **.**

Height: ……**m. Weight:** …… **Kg. BMI:** …… **Kg/m2**

Sys BP: ……**mmHg. Dias BP:** ……**mmHg.**

Smoker: Yes ( ) No ( ) Ex-smoker: Yes ( ) No ( ) For ……. **Years.**

Passive smoker: Yes ( ) No ( ) For ……. **Years.**

Do/ Did You Have:

Hypertension ( ) Heart failure ( ) Retinopathy ( ) Liver disease ( ) Acute or chronic renal failure ( ) Nephrotic syndrome ( ) Hypothyroidism ( ) Hyperthyroidism ( )

Other diseases:…… .

Do you take:

Metformin ( ) Insulin ( ) Statins ( ) fibrates ( ) Niacin( ) Ezetimibe ( ) A C E inhibitor( ).

Other drugs or surgery:…… .

FBS:……mg/dl. HBA1c:…….. %. TC:……. mg/dl. HDL:……. mg/dl. LDL:……. mg/dl. TG:…….. mg/dl.

المقدمة

داء السكري (DM) هو على الارجح واحدة من أقدم الأمراض المعروفة للناس، ويعرف مرض السكري بأنه مرض مزمن سببه نقص في إفراز الأنسولين من البنكرياس، أو عدم فعالية الأنسولين المنتج. ، الأمر الذي يؤدي إلى ارتفاع السكر في الدم الذي بدوره يؤدي الي الاضطرابات في ايض الكربوهيدرات والدهون و البروتين. كما انه كلما تطور المرض سبب تلف الأنسجة أو الأوعية الدموية مسببه مضاعفات السكري الحادة مثل اعتلال الشبكية، اعتلال الأعصاب، اعتلال الكلية ومضاعفات القلب والأوعية الدموية.

انزيمات نواقل الجلوتاثين هي عائلة من الإنزيمات التي تلعب دورا هاما في إزالة السموم وذلك بتحفيز تصريف العديد من المركبات باتحادها مع الجلوتاثين المختزل ، حتى أنها تدافع عن خلايا ضد طائفة واسعة من المواد الكيميائية السامة والمؤكسدة.

زيادة انتاج المواد المؤكسدة تزداد بنسبة عالية عند ارتفاع المزمن من السكر في الدم، حيث ارتفاع المواد المؤكسدة يسبب فشل كامل في وظيفة خلايا البيتا من البنكرياس ، ومقاومة لفعالية الانسولين المنتج ، وكذلك يسبب احتمالية تصلب الشرايين.

لذللك وظيفة انزيمات نواقل الجلوتاثين مهمة جدا لطرد المواد المؤكسدة في مرضي السكري، أي خلل في عمل هذه الانزيمات لسبب خلل جيني علي مستوي الجين قد يزيد احتمالية الاصابة بم هذه الدراسة صممت لتقييم دور انزيم ناقل الجلوتاثين ب1 في داء السكري النوع 2 بالإضافة إلى ارتباطه مع قياسات السكر والوزن والضغط وقياسات الدهون.

المرضي وطرق الفحص

في هذه الدراسة تم الحصول على مجموع 67 عينة من المرضى الليبيين المصابين بداء السكري النوع 2 من مركز بنغازي لتشخيص وعلاج مرض السكري. كما تم الحصول على 31 عينه من أشخاص اصحاء ومطابقين للمرضى من حيث العمر و الجنس من المعهد العالي للمهن الطبية، بنغازي لتكون بمثابة الضوابط.

تم الحصول على المعلومات السريرية والتاريخ الطبي من خلال المقابلات مع المرضى و الحالات الضابطة. تم سحب عينات من الدم الوريدي من جميع المشاركين بعد 10 ساعات على

الأقل من الصيام، ومن ثم تحليل مستوى السكر في الدم ، والسكر التراكمي، والكولسترول الكلي، والدهون الثلاثية، والدهون ذات الكثافة العالية باستخدام المحلل الاتوماتيكي بتقنيات أنزيمية. أما بالنسبة للدهون ذات المنخفضة العالية فقد تم احتسابها وفقا لمعادلة Friedewald. فقد تم تحليل البيانات باستخدام spss النسخة 18، وقيمة p اصغر من 0.05 ذو دلالة إحصائية.

تم تضخيم الجين المنتج لأنزيم ناقل للجلوتاثين بعد ان قمنا باستخلاص الجين بالكامل من عينات الدم تم تضخيم الجين المنتج لأنزيم ناقل للجلوتاثين باستخدام(PCR) ثم تم نقع نواتج التضخيم طوال الليل مع انزيم القص(ALW261) وبعدها تم انزالهم علي 2.5% من الجل باستخدام (electrophoresis) .

النتائج

بعد ان تم تحليل البيانات وجود انه يوجد اختلاف واضح في قياسات السكر والسكر التراكمي والدهون وقياس body mass index والضغط ما بين المجموعتين($p\leq0.05$).

حيث ان وجوده نسبة الجين المتطفر(G allele)كانت اعلي من الجين الطبيعي(A allele) في المجموعة الضابطة اكثر مما يليها في مجموعة السكري .

الخاتمة والتوصية

هذا يوضح لنا ان جين (GSTP1) لا يلعب دور هام في مرضي السكر، حيث نحن نوصي بدراسة الاشكال الأخرى للجين(GSTT&GSTM) لنري اذا كان هناك أي خلل جيني متعلق بمرضي السكري.