Epigenetic Regulation of Adipocyte genes in Type 2 Diabetes

BY

Anum Hubab Hashmi

NUST201361708MASAB92513F

Atta-Ur-Rahman School of Applied Biosciences,

National University of Sciences & Technology.

Islamabad, Pakistan.

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Anum Hubab Hashmi

NUST201361708MASAB92513F

Supervised by: Dr. Attya Bhatti

Atta-Ur-Rahman School of Applied Biosciences

National University of Sciences & Technology.

Islamabad, Pakistan.

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I dedicate this work to my dearest Grandmother, my Ami and Abu for their unconditional love and prayers.

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List of Acronyms

Akt	Protein Kinase B
BLAST	Basic Local Alignment Search Tool
BLAT	BLAST-like Alignment Tool
BMI	Body Mass Index
C3G	Guanylnucleotide-exchange protein
cAMP	Cyclic Adenosine monophosphate
CAP	Cbl Associated Protein
Cbl	Casitas B-Lineage Lymphoma
CFD	Complement Factor D
CpG	Cytosines following guanines
Ct	Cycle Threshold
DEPC	Diethylpyrocarbonate
Dnmt	DNA methyltransferases
FFA	Free Fatty Acids
FPG	Fasting Plasma Glucose
GDP	Guanosine diphosphate
GLUT4	Glucose Transporter 4
GTP	Guanosine triphosphate
HbA1c	Glycated Hemoglobin
IFG	Impaired Fasting Glucose
IGF1	Insulin-like Growth Factor 1
IGT	Impaired Glucose Tolerance
IκB	Inhibitor kappa B
IL-1β	Interleukin-1 β
IRS	Insulin Receptor Substrate
LDL	Low Density Lipopolysaccharide

MCP	Monocyte Chemoattractant Protein
mRNA	Messenger Ribonucleic Acid
NEFA	Non-Esterified Fatty Acid
NIDDM	Non-Insulin Dependent Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
PDK1	Phosphoinositide-dependent Kinase 1
PI(3)K	Phosphatidyl Inositol (3) Kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein Kinase C
PPARγ	Peroxisome Proliferator-activated Receptor
RNA	Ribonucleic Acid
RPG	Random Plasma Glucose
SGK	Glucocortocoid-inducible Kinase
SH2	Src-Homolgy 2
SNP	Single Nucleotide Polymorphism
T2DMM	Type 2 Diabetes Mellitus
TNFα	Tumor Necrosis Factor alpha
UCP-1	Uncoupling-protein 1

ABSTRACT

Type 2 Diabetes Mellitus (T2DMM) is a complex metabolic disorder characterized by hyperglycemia due to insulin insensitivity and insufficient synthesis of insulin by pancreatic β-cells. Tumor Necrosis Factor α (TNFα), one of the major pro-inflammatory cytokines released by adipose tissue, is largely associated with insulin resistance by attenuation of insulin signaling. Individuals with obesity and T2DM show a noticeable increase in the levels of TNFα. Therefore, TNFα serves as an important culprit in progression of T2DM and acts as a significant target for therapy in T2DM. This study was designed to correlate the changes in expression of TNFα with DNA methylation status of its promoter. Therefore, expression of TNFα and changes in DNA methylation patterns at its promoter region were analyzed between control and T2DM individuals. The results depicted reduced DNA methylation at the TNFα promoter indicating enhanced transcription and thus, elevated levels of TNFα in individuals with T2DM.

INTRODUCTION

1.1 Background

Diabetes is a complex metabolic disorder characterized by hyperglycemia due to insufficient production, signaling and ineffective action of insulin in the body (Cohen and Horton, 2007). Diabetes is divided into two main categories: Type 1 Diabetes is immune-mediated where immune cells attack pancreatic β-cells resulting in complete loss of insulin synthesis (Devendra *et al.*, 2004). Whereas, Type 2 Diabetes or adult-onset diabetes is associated with inability of target cells to respond to insulin and progressive dysfunction of β-cells leading to insufficient insulin synthesis (Kohei, 2010). Incidence of diabetes is on the rise globally and 170 million individuals are estimated to be effected by it, 90% of which are diagnosed with T2DM (Stumvoll *et al.*, 2005) and 5-10% comprise of Type 1 Diabetics (Daneman, 2006). Type 2 Diabetes is a polygenic disorder where multiple genes are involved in impaired insulin secretion and development of insulin resistance. It is caused by both genetic i.e. familial disposition and acquired environmental factors like lack of physical exercise, weight gain, overeating and stress (DeFronzo, 1988; Kohei, 2010).

Apart from hyperglycemia, individuals with T2DM suffer from various symptoms including polyuria (frequent urination), polydipsia (thirst), polyphagia (overeating) and loss in weight (CDC, 2012). T2DM gives rise to many serious and severe complications including cardiovascular diseases, retinopathy leading to potential loss of vision, nephropathy leading to kidney failure, limb amputation and neuropathy. Moreover, risk of stroke and cardiovascular morbidity is two to four times greater in diabetic individuals than non-diabetics (Hotta *et al.*, 2000; Zimmet *et al.*, 2001). These complications associated

with T2DM are the cause of major socioeconomic burden on both developing and developed nations (Wei *et al.*, 1998).

1.2 Epidemiology

The global incidence of diabetes mellitus has doubled over the past three decades (Danaei *et al*., 2011) owing to increasing population, aging, urbanization, obesity and lack of physical activity (Zimmet *et al.*, 2001). According to an estimate in 2010, 285 million people were affected with diabetes mellitus (Shaw *et al.*, 2010), 90% of which had T2DMM (Zimmet *et al.*, 2001). It has been projected that by 2030, the number of individuals affected with diabetes mellitus will rise to 439 million (Figure 1.1) (Chen *et al.*, 2011), which constitutes 7.7% of the world adult population between ages 20-79 years (Shaw *et al.*, 2010).

Few decades ago, major burden of diabetes was present in developed nations. However, 80% of the diabetics now reside in developing and less developed countries (Shaw *et al.*, 2010). Asia has now become the epicenter of diabetes as 5 countries out of 10 estimated to have the highest number of diabetics by 2030 are in Asia (Pakistan, India, China, Indonesia and Bangladesh) (Wild *et al.*, 2004). Besides Asia, Gulf regions of the Middle East and Africa are also another hot spot region for diabetes (Mbanya *et al.*, 2010). Also, unlike the general perception, diabetes is not less prevalent in rural population as compared to urban population of developing countries (Shaw *et al.*, 2010). The prevalence gap between the two is being minimized due to increasing urbanization and its associated changes in lifestyle.

T2DMM which is usually considered as an ailment of adults, is now becoming more prevalent in young adults, adolescents and though less often but in children as well (Pinhas-Hamiel and Zeitler, 2005). Pediatric T2DMM constitutes more than 80% of diabetes mellitus cases in adolescents of various ethnic groups like American Indian, Asian, and Pacific Islander population (Kitagawa *et al.*, 1998).

Ethnic groups like Native Americans, Indigenous Australian population, African Americans, Hispanics, Pacific Islanders and Asians have been identified as high risk groups for T2DM (Liese *et al*., 2006). For example, among the general Australian population, Indigenous Australian adults are at a six times higher risk of developing T2DM (Craig *et al.*, 2007).

Pakistan ranks at sixth position among the top ten countries with highest number of individuals with diabetes (Wild *et al.*, 2004). Population-based surveys conducted in three provinces of Pakistan found that 10% of adult individuals suffer from T2DM and about the same number have impaired glucose tolerance (Shera *et al.*, 1999a; Shera *et al.*, 1995; Shera *et al.*, 1999b).

1.3 Pathophysiology of Type 2 Diabetes

Type 2 Diabetes is a complex disorder of impaired carbohydrate and fat metabolism. It is characterized by impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). The abnormalities in glucose metabolism occur due to an imbalance between insulin sensitivity and insulin secretion from β-cells (Scheen, 2003). This imbalance consequently impairs glucose metabolism leading to hyperglycaemia. Insulin insensitivity is also associated with lipid accumulation in liver, muscle and visceral adipose tissue. Lipid accumulation in target tissues (liver and muscle) also is a cause of their insensitivity to insulin (D'Adamo and

Caprio, 2011). Apart from muscle, liver and β-cells (the triumvirate, Fig. 1.2) (DeFronzo, 1988), adipocytes, gastrointestinal tract, kidney, brain and α-cells have also been found to play important roles in glucose intolerance in T2DM. Impairments in glucose metabolism occurs due to gradual dysfunction of β-cells along with insulin insensitivity.

1.3.1 β-cell function

β-cell dysfunction occurs gradually over time, hence making T2DM a progressive disorder (DeFronzo, 2009). Several studies have demonstrated that T2DM only occurs when β-cells are no longer capable of appropriately responding to peripheral insulin resistance by increasing insulin secretion. The ability of the β-cells to release insulin in response to insulin resistance depends upon their mass and secretory potential (Kahn *et al.*, 2006) which is influenced by genetic and environmental factors (Lyssenko *et al*., 2008).

1.3.2 Insulin Resistance

Although the pathogenesis of T2DM is not completely understood, it is clear that insulin resistance plays a significant role in its progression. Several cross-sectional and longitudinal studies have shown that insulin resistance develops 10-20 years before the onset of type 2 diabetes and thus, has been agreed upon as a most important predictor of T2DM (Shulman, 2000). As a result of insulin resistance, β-cells have to go through the strenuous work of increasing insulin secretion in order to compensate for the peripheral insulin insensitivity, therefore affecting the functioning efficiency of β-cells. The precise mechanism by which insulin insensitivity leads to β-cell failure remains unknown. However, lipotoxicity, which plays a strong role in developing insulin insensitivity, is believed to be a cause of β-cell gradual failure as well (DeFronzo, 2009; Kahn *et al.*, 2006).

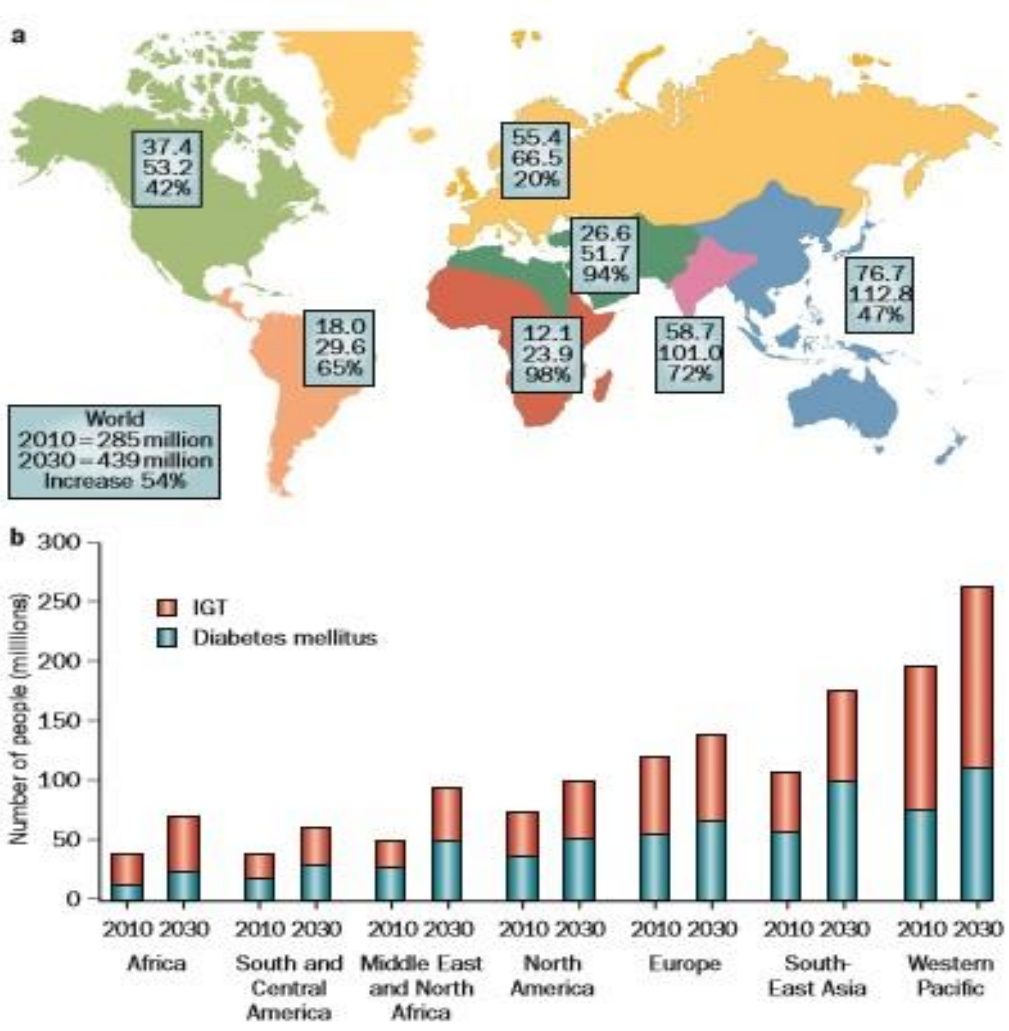

Figure 1.1 Global Projections for Diabetes mellitus epidemic: 2010-2030 (a) The top and middle values depict the number of diabetic individuals (in millions) in 2010 and 2030 respectively; the bottom value is the percentage increase from 2010 to 2030. (b) Number of individuals with diabetes mellitus and IGT among adults aged between years 20-79 for the years 2010 and 2030 (Chen *et al.*, 2011).

1.4 Criteria for Diagnosis of Type 2 Diabetes

To diagnose T2DM, blood tests are often used in which blood is drawn from an individual and sent to a laboratory for testing. Testing can enable health care providers to diagnose T2DM and prediabetics and help them to control and prevent complications to occur and delay onset of the disease, respectively. Various tests are used for the T2DM diagnosis including (a) A1C or HbA1c which is a glycohemoglobin or hemoglobin test which measures the thickness of sugar bound to hemoglobin in red blood cells over the period of past three months (life span of a red blood cell). Higher the blood glucose levels in a person, thicker the sugar coat on the red blood cells, (b) Fasting Plasma Glucose (FPG) which is a simple blood test done after several hours of fasting to help diagnose diabetes and pre-diabetes, (c) Oral glucose tolerance test (OGTT) which is used to measure the body's ability to use glucose which is body's main energy source.

Random plasma glucose (RPG) test could also be used for diagnosis of diabetes. If RPG is found to be 200 micrograms per deciliter or above and the individual also shows symptoms for diabetes, the individuals are diagnosed to diabetics.

Classic symptoms of T2DM include polyuria, polydipsia, polyphagia, weight loss, vision blurring, inability to heal wounds and yeast infections (CDC, 2012). The diagnostic criteria of T2DM as per American Diabetes Association is given in Table 1.1.

Table 1.1 Criteria for diagnosis of diabetes mellitus

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h plasma glucose 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dl (11.1 mmol/l).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing (Association, 2011)

1.5 Risk factors for Type 2 Diabetes

1.5.1 Environmental Factors

(a) Obesity

Obesity is growing into a global epidemic and alarming trends are being seen worldwide. In USA, only about a third of adults are considered to be of normal weight (WHO, 2000). Obesity is defined as over accumulation of adipose tissue which affects physical, psychological, social health and well-being (Naser *et al.,* 2006). It also plays a significant role in development of metabolic disorders. Under normal circumstances, adipose tissues mediate metabolic regulation by releasing non-esterified fatty acids (NEFAs), glycerol, hormones and various inflammatory cytokines (Shoelson *et al.*, 2006). In obese individuals, production of inflammatory cytokines from adipose tissues rises which eventually causes insulin resistance in target tissues. Increase in release of NEFAs from adipose tissues is a major contributing factor in development of insulin resistance as its levels have been found to increase markedly in non-insulin dependent diabetes mellitus (NIDDM) (Reaven *et al.,* 1988).

In wake of insulin resistance, the target tissues do not respond to insulin and therefore uptake of glucose from blood doesn't occur and thus, results in high blood glucose levels. To counteract increased blood glucose and insulin insensitivity, pancreatic β-cells increase insulin secretion into the bloodstream in order to maintain normal glucose tolerance (Perley and Kipnis, 1966). For an obese and insulin-resistant individual to develop T2DM, the release of insulin from β-cells must not be enough to compensate for its lack of sensitivity and action. Insulin release from β-cells is compromised due to their progressive failure

which occurs in individuals highly susceptible to T2DM despite normal glucose levels (Kahn, 2001). In obesity, NEFAs are released from adipose tissues into the bloodstream and induce insulin insensitivity in target organs (Kahn *et al.*, 2006).

Distribution of body fat is a major determinant of insulin sensitivity. Though simple obesity is associated with insulin resistance, in lean individuals its severity depends upon distribution of body fat. Lean individuals with peripheral fat are more sensitive to insulin action than those with central fat distribution i.e. in abdomen and chest areas (Kahn *et al.*, 2006).

(b) Physical Inactivity

Various studies have identified physical activity to be strongly associated with development of type 2 diabetes (Fretts *et al.*, 2009; Jeon *et al.*, 2007). Physical activity has been found to lower the risk of T2DM and to delay its onset in individuals with impaired-glucose tolerance (IGT) who are highly susceptible to T2DM (Pan et al., 1997). Increased physical activity tends to vary BMI and therefore, the subsequent reduction in weight lowers the risk of diabetes in individuals with high risk of the disease (Hamman et al., 2006). Weight reduction due to physical activity subsequently improves insulin sensitivity and reduces resistance in target organs leading to normal glucose tolerance.

(c) Hypertension

It has been known that hypertension is prevalent in individuals with obesity and type 2 diabetes, both states being insulin resistant (Ferrannini, 1995). After the discovery that hypertension in itself is an insulin resistant state (Ferrannini *et al.*, 1987), it was made a part of metabolic syndrome. However, insulin resistance in hypertensive state is limited to glucose metabolism and only affects peripheral tissues but not the liver (Ferrannini *et al.*,

1987). Research studies have shown that there exists an association between hypertension and T2DM. Evidence suggests that C-reactive protein, an inflammatory marker, has elevated levels in both T2DM (Hu *et al.*, 2004) and hypertension (Blake *et al.*, 2003). Endothelial dysfunction is another link between hypertension and T2DM as the former has been found to be associated with impaired nitrogen oxide-mediated vasodilation of blood vessels (Gokce et al., 2001). Moreover, studies have shown that markers of endothelial dysfunction are also present in onset of diabetes. A recent study demonstrated that use of valsartan, an angiotensin II blocker with antihypertensive properties, in patients with impaired glucose tolerance and cardiovascular disorder, reduced the incidence of T2DM by 14% (McMurray et al., 2010).

(d) Dietary Patterns

Type of food intake has a major impact on an individual's health. Likewise, several studies have shown that dietary patterns are largely associated with development of T2DM. A number have cohort studies have shown that individuals consuming more of red meat, processed food, high-fat dairy products, sweets and desserts have a high incidence of T2DM than individuals consuming 'prudent' diet which includes fruits, vegetables, poultry and whole grains (Liese *et al.*, 2009; Montonen *et al.*, 2005; van Dam *et al.*, 2002). It has also been found that consumption of foods with high glycemic index i.e. with higher ability to affect blood sugar and insulin levels, is associated with incidence of T2DM (Schulze *et al*., 2004). Foods rich in rapidly absorbed carbohydrates, saturated fats and with low dietary fiber tend to increase risk of T2DM. It has been recommended that in order to minimize risk of T2DM mellitus, consumption of food with low-glycemic index, high fiber content,

minimally processed whole-grain should be increased as diet such as this would reduce glycemic and insulinaemic responses hence, lowering risk of T2DM (Hu *et al.,* 2001).

1.5.2 Genetic Factors

Type 2 diabetes is a multifactorial disorder which develops due to a complex interplay of genetic, epigenetic and environmental factors. Type 2 Diabetes Mellitus is an inherited disorder as with individuals having a positive family history being more susceptible to acquire the disease. Lifetime risk of T2DM in individuals with one affected parent is 40% and almost 70% in those with both parents affected (Kobberling and Tillil, 1982) . Moreover, it has also been found that concordance rate of T2DM in monozygotic twins is up to 70% while in dizygotic twins it is only 20-30% (Kaprio *et al*., 1992).

Several studies including Genome-wide Association Studies (GWAS) have identified T2DM to be a complex, multifactorial and most importantly polygenic disorder. Polygenic refers to the contribution of multiple genetic regions in development of a disease. Genetic factors interact with each other and with environmental factors giving rise to T2DM pathogenesis. However, the genetic variants associated with T2DM have been found to be not penetrant enough to cause the disease on their own without the contribution of environmental factors. Heritability of T2DM has been found to range from 30% - 70% in family or twin studies depending on the age of diabetes onset and glycemic status of the individual (Almgren *et al.*, 2011; Poulsen *et al.*, 1999). Familial linkage-analysis studies during last 10 years have identified few genetic loci to be associated with type 2 diabetes. These loci include CAPN10 (Hanis *et al*., 1996), ADIPOQ (Vionnet *et al*., 2000), HNF4 (Silander *et al*., 2004), TCF7L2 (Grant *et al*., 2006), ENPP1. Moreover, association of TCF7L2 and HNF4 was also confirmed by the Genome-Wide Association Analysis

(GWAS). TCF7L2 has been identified to be a T2DM-susceptibility gene having a SNP that has been repeatedly demonstrated to be strongly associated with T2DM having an Odds Ratio of 1.50 (Grant *et al.*, 2006). TCF7L2 codes for a transcription factor involved in Wnt signaling which controls various cellular mechanisms in different tissues including those playing a role in metabolism. It has been reported that TCF7L2 might play an important regulatory role in insulin synthesis and secretion from pancreatic β-cells thus making it an important player in pancreatic phenotype but not in insulin resistance or liver abnormalities (Saxena *et al*., 2010).

1.5.3 Epigenetic Factors

Besides environmental and genetic factors, it has also been established through various research studies that epigenetics plays a significant role in disease progression. The word 'epi' is of Greek origin and means 'above, over' thus referring to the fact that epigenetic changes occur 'over' the DNA and not on the primary DNA sequence (Bramswig and Kaestner, 2012). Epigenetics is defined as heritable and reversible changes in gene expression owing to the state of chromatin without any alteration of the underlying DNA sequence (Turner, 2002). Chromatin is a complex of DNA, histone proteins and several DNA or protein binding factors which not only help in DNA packaging but also act as transcriptional regulators. Transcription in eukaryotes is a precisely regulated process which is controlled by the degree of chromatin accessibility to transcription factors. Consequently, chromatin accessibility is determined by the state of structural complexity of DNA and histone proteins thus determining the regions of transcriptional repression and activation (Bird, 2007). The structural state of chromatin in turn determines the ability of transcription machinery to access the DNA at a particular locus and transcribe it (Jenuwein

and Allis, 2001). Environmental factors have been known to influence the epigenetic landscape as well, thus playing a significant role in various human diseases. Effect of environment on epigenetics has been proposed to consist three steps: the first environmental signal initiates a second intracellular signal which establishes the precise chromosomal location where the epigenetic modification will occur, and the third signal which sustains the very modification (Berger *et al.*, 2009). For example, early exposure to hyperglycaemia leads to complications that develop in the later stages of disease despite of improved hyperglycemic control (Keating and El-Osta, 2012). Another environmental factor like increased food intake could lead to obesity (BMI>30) which is an important risk factor for diabetes. Studies during Dutch hunger famine have shown that intrauterine malnutrition and low birth weight when followed by increased BMI in childhood increases the susceptibility to develop metabolic syndrome which in turn leads to an increased risk of T2DM (Kassi *et al.*, 2011). Hence, various studies have proven that epigenetic factors indeed play a major role in disease pathogenesis.

1.6 Treatment Options

Type 2 Diabetes being a complex disease involving both genetic and environmental factors, can be managed by both drugs and by changes in lifestyle.

1.6.1 Management without Drugs

The first line of treatment for T2DM is diet control, weight management and physical activity. A balanced diet constituting primarily of non-starch whole grain carbohydrates which are high in fiber content and low in saturated fats helps in control of slightly increased blood glucose levels (Steyn *et al.*, 2004). High caloric food intake and built up

of excess adipose tissue induces insulin resistance and leads to decreased glucose uptake by cells and reduced glycogen synthesis. Increase in the levels of saturated fats has been associated with elevated fasting insulin levels and reduced insulin sensitivity, whereas non-saturated fats show an opposite effect. Increased BMI and waist to hip ratio has shown a positive correlation with risk of T2DM (Steyn *et al.*, 2004). Moreover, various studies have shown that diets high in carbohydrates and low in fat content result in improved glycemic control and reduction in low density lipids (LDL) as compared to diet with more fat and less carbohydrates. Also exercise and increased physical activity has also shown improvement in regulation of plasma glucose levels and improvement in insulin sensitivity.

1.6.2 Drug Therapies

There are various drugs available which are used for management of T2DM as listed below:

- Metformin: It is used to lower levels of blood glucose by suppressing hepatic glucose production, increasing peripheral glucose uptake and insulin sensitivity (Lechleitner, 2011)
- Thiazolidinedione: This medication works to increase the levels of receptor molecules, especially peroxisome proliferator-activated receptors which are nuclear receptor and acts as transcription factors for various genes involved in fat and glucose metabolism. However, thiazolidinediones show some adverse effects including increased edema and cognitive heart failure (Saraf *et al.*, 2012)
- Dipeptidyl peptidase-4 inhibitors: Work by blocking DPP-4, which reduces glucagon production and increases insulin production thereby reducing blood glucose levels (Lechleitner, 2011).

- α-Glucosidase Inhibitors: This medication is used to decrease glucose digestion by competitive inhibition of enzymes involved in catalysis of carbohydrate digestion in the intestine thus lowering postprandial blood glucose (Lechleitner, 2011).
- Sulfonylureas: A class of drugs which stimulates glucose-dependent insulin secretion. However, it can cause hypoglycemia as a potential side effect.
- Repaglinide: This drug is administered prior to intake of a meal in order to stimulate insulin secretion though with a shorter half-life as compared to sulfonylureases.

1.6.3 Insulin Therapy

As progressive failure of β-cells occurs in T2DM, therefore over a period of time pancreas can no longer synthesize and secrete insulin via the use of oral medication. As a result, the patient is then recommended to be administered with insulin injections. At the start, insulin therapy normally starts with one injection per day plus the use of oral medication. Multiple injections are administered when blood glucose is no longer controlled with one injection.

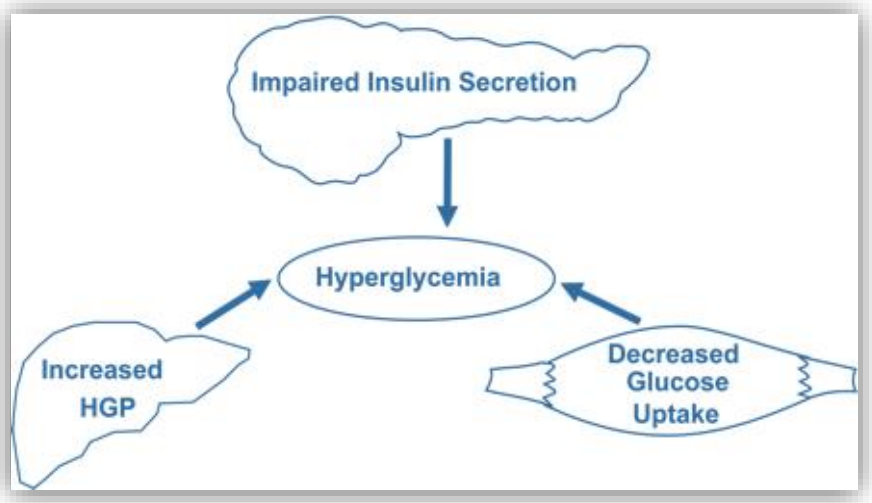

Figure. 1.2 Pathogenesis of Type 2 Diabetes: the triumvirate. Insulin resistance in muscle and liver and impaired insulin secretion represent the core defects in type 2 diabetes (DeFronzo, 2009)

1.7 Aims and Objectives

The aim of this study was to find out if epigenetic markers, DNA methylation in particular has a role to play in changes in expression of TNFα during T2DM pathogenesis and development.

I. To evaluate differences in expression of TNFα between patients with T2DM on insulin treatment, T2DM patients without insulin treatment and non-diabetic controls.

II. To find differences in the methylation status of TNFα promoter at two loci between T2DM patients and control individuals.

III. To correlate the expression of TNFα with the status of DNA methylation at its promoter.

Chapter 2

Review of Literature

Type 2 Diabetes is a complex disorder which has now become an epidemic due to global increase in its occurrence. It is characterized by an early phenomenon of insulin insensitivity which is also related to obesity, gradual β-cell failure which occurs before the onset of clinical hyperglycaemia. Several mechanisms leading to T2DM have been proposed including an increase in NEFAs, adipokines, inflammatory cytokines, lipotoxicity, glucotoxicity, amyloid formation for β-cell dysfunction. Several genetic factors have also been reported to play a significant role in pathogenesis of T2DM. These include genes for Calpain 10, peroxisome proliferator-activated receptor γ, and insulin-receptor substrate 1 among others. Epigenetic factors have also been identified to play a very significant role in development of T2DM. Epigenetics refers to reversible and heritable changes in gene expression without any alteration of the primary DNA sequence. Changes in gene expression tend to owe to the state of the chromatin of the particular locus. Therefore, the status of DNA methylation and histone modifications can control the state of chromatin folding or unfolding and subsequently regulating gene expression.

2.1 Insulin Signaling Pathways

Blood glucose levels remain within a narrow range of 4 to 7mM despite varying lengths of periods of feeding and fasting. This control of blood glucose levels is due to a balance between glucose absorption from the intestine, production from liver and uptake and metabolism by muscle and fat. This balance is dictated by the ability of insulin, the most potent physiological anabolic hormone, to regulate glucose uptake in muscle and fat as

well as its ability to reduce its production from the liver (Figure 2.1). Insulin enhances glucose uptake via glucose transporters GLUT4 recruited into the plasma membrane as a result of insulin signaling. Apart from regulation of blood glucose, insulin also stimulates cell growth and differentiation, storage of carbohydrates, lipids and proteins in fat tissues, muscle and liver (Saltiel and Kahn, 2001). Any abnormality in insulin function results in severe dysregulation of all these processes resulting in metabolic dysregulation which gives rise to several metabolic disorders. Therefore insulin serves as the most significant player in maintaining metabolic homeostasis.

2.1.1 Insulin Receptor Signaling

The insulin receptor belongs to the subfamily of receptor tyrosine kinases which also includes Insulin-like Growth Factor (IGF)-1 and Insulin receptor-related receptor (Patti and Kahn, 1998). When insulin binds to the α-subunits, it induces the transphosphorylation of one β-subunit by another at specific tyrosine residues. Transphosphorylation results in an enhanced kinase activity of the protein (Lietzke *et al*., 2000; Myers *et al*., 1992). Autophosphorylation also occurs at other tyrosine residues located within the juxtamembrane region and intracellular tail of the receptor (Chang *et al.*, 2004). Activated insulin receptor then phosphorylates tyrosine residues on downstream signaling molecules and substrates which include insulin receptor substrate family (IRS1 through 4), IRS5/DOK4, IRS/DOK5, Gab-1, Cb1, APS, and Shc isoforms (Saltiel and Kahn, 2001). Phosphorylated tyrosines of these substrate molecules act as docking sites for proteins containing the Src-Homology-2 (SH2) domain, for example the p85 regulatory subunit of PI3K (Saltiel and Kahn, 2001).

2.1.2 The Phosphatidylinositol-3-Kinase Pathway

Phosphatidylinositol-3-kinase (PI(3)K) plays a fundamental role in insulin's metabolic and mitogenic actions. Studies have shown that knockout or inhibition of PI3K results in disruption of most metabolic activities of insulin including glucose uptake, glycogen and lipid synthesis (Shepherd *et al.*, 1995). PI(3)K consists of a p110 catalytic and p85 regulatory subunits. The p85 regulatory subunit has two SH2 domains that recognize and bind to the phosphorylated tyrosine residues of IRS proteins (Shepherd *et al.*, 1995) leading to the activation of PI(3)K enzyme. Once activated, it catalyzes the phosphorylation of phosphoinositides on three positions to produce phosphatidylinositol 3,4,5-triphosphate (PIP_3) which in turn alters the activity or subcellular localization of numerous proteins (Lietzke *et al.*, 2000; Shepherd, 2005).

PIP_3 binds to the pleckstrin homology (PH) domain of various proteins including signaling molecules, enzymes, and adapter proteins and activates/recruits them to the plasma membrane (Lietzke *et al.*, 2000). These proteins include Ser/Thr PDK1 which subsequently phosphorylates and activates various downstream signaling molecules including Akt1 through 3, protein kinase C (PKC) and glucocorticoid-inducible kinase (SGK) (Mora *et al.*, 2004). PIP_3 also appears to mediate the translocation of Akt to the plasma membrane via its PH domain (Corvera and Czech, 1998). This cascade downstream signaling eventually results in translocation of GLUT4 from intracellular sites to the plasma membrane to allow for uptake of glucose into the cell (Figure 2.2).

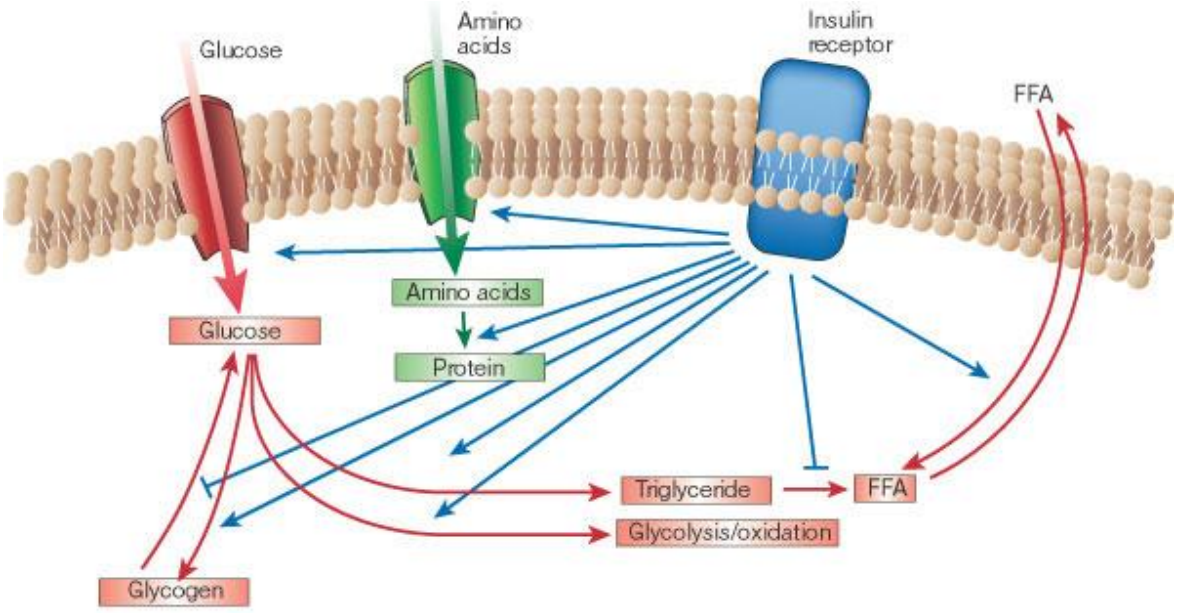

Figure. 2.1 The regulation of metabolism by insulin. Insulin is the most potent anabolic hormone known, and promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression or activity of enzymes that catalyze glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyze degradation.(Saltiel and Kahn, 2001)

2.1.3 Insulin Signaling from Lipid Rafts

Several studies have shown the existence of another insulin signaling pathway localized within the lipid rafts in the plasma membranes of cells. These lipid rafts are regions rich in sphingolipids, cholesterol, glycosylphosphotidylinositol (GPI)-anchored proteins, glycolipids (Smart *et al.,* 1999). It has been shown that some of the insulin receptors reside in these lipid raft domains via interaction with a lipid raft protein caveolin (Gustavsson *et al.,* 1999; Kimura *et al.,* 2002; Parpal *et al.,* 2001). Activation of an insulin receptor activates the adapter protein APS which exists as a homodimer and has both SH2 and PH domains (Baumann *et al.,* 2000; Kimura *et al.,* 2002).

Once activated, APS interacts with the three phosphotyrosines on the activation loop of the insulin receptor via its SH2 domain (Hu *et al.,* 2003). Each β-subunit of the insulin receptor interacts with one APS homodimer, thereby allowing the receptor to recruit two APS homodimer at the same time (Hu *et al.,* 2003). Upon binding to the receptor, a C-terminal tyrosine of APS is phosphorylated and act as a docking site for Cbl protein which binds via its SH2 domain and is subsequently phosphorylated at three tyrosine residues (Liu *et al.,* 2002). c-Cbl and Cbl-b are proto-oncogenes which are activated as a result of activation of insulin receptors within lipid rafts (Ribon and Saltiel, 1997). Cbl is associated with an adapter protein called Cbl associated protein (CAP) and both of these get recruited to APS once its activating tyrosine phosphorylation occurs. CAP binds to the proline-rich sequences of Cbl via its C-terminal SH3 domain (Ribon *et al.,* 1998a). Expression of CAP correlates with insulin-sensitivity of the cell and is predominantly expressed in insulin-sensitive tissue (Ribon and Saltiel, 1997). Also, activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ), a receptor for thiazolidinedione

class of insulin-sensitizing drugs, or use of PPAR γ agonists leads to increased expression of CAP (Ribon *et al.,* 1998b; Ribon and Saltiel, 1997). Upon phosphorylation, the Cbl–CAP complex translocates to lipid rafts in the plasma membrane, mediated by the interaction of the SoHo domain of CAP with the protein flotillin (Baumann *et al*., 2000). Translocation of Cbl recruits the adapter protein CrkII to the lipid rafts. CrkII forms a constitutive complex with the guanyl nucleotide-exchange protein C3G. After translocation, C3G comes into proximity with the G protein TC10, and catalyzes the exchange of GTP for GDP, resulting in activation of TC10 (Chiang *et al*., 2001). Once activated, TC10 is thought to provide a second signal to the GLUT4 protein which translocates to the plasma membrane and allows for glucose uptake by the cell (Chiang *et al.*, 2001).

2.2 Role of Adipocytes in Pathogenesis of Type 2 Diabetes

Adipose tissue acts as a site for fat storage. A lot of research is now focused on adipose tissue after the establishment of the part it plays in obesity and associated disorders of T2DM mellitus and cardiovascular disease. Adipose tissues have an integral role in metabolic homeostasis. Besides, regulation of fat mass and metabolic homeostasis, adipocytes also regulate blood pressure, immune response, and bone mass, reproductive and thyroid function (Trayhurn, 2005). Adipocytes store fats as triglycerides and break them down into free fatty acids (FFA) and glycerol during times of energy deficiency when glucose is limiting. Triglycerides release more energy per unit mass as compared to carbohydrates.

White adipose tissue has long been recognized as the main site for the storage of excess of energy (Rosen and Spiegelman, 2006). Excess energy is stored within white adipose tissue as highly concentrated triglycerides in the form of a single large lipid droplet. These droplets are associated with proteins which enable their sequestration and efficient storage. These stored triglycerides are hydrolyzed upon need by lipases in a process known as lipolysis in order to release free fatty acids into the circulatory system from where they enter the cells and are oxidized by mitochondria to release energy. In contrast to white adipose tissue, brown adipose tissue is specialized for non-shivering thermogenesis, which is an adaptation of cold climate. Brown adipose tissue, contain smaller and multiple lipid droplets which allows for their rapid lipolysis in times of need. These adipocytes also contain a higher number of mitochondria and a high content of uncoupling-protein 1 (UCP-1) which allows heat generation from the energy produced by fatty acid oxidation (Guilherme *et al.*, 2008). Brown fat reserves are predominantly present in infants and some evidence from recent studies suggests that some of these depots might be present in dispersed state in adults (Nedergaard *et al.*, 2007).

Increasing incidence of obesity worldwide has made it evolve into a modern biomedical problem (Nedergaard *et al.*, 2007). A classic definition of obesity is the excess of adipose tissue in the body (Taton, 1981). Increase in adipose tissue in obese condition is associated with its compromised function and low-grade inflammation (Hajer *et al.*, 2008). Individuals having a BMI of more than 30 kg per m^2 in particular are defined as obese (Guilherme *et al.*, 2008). Obesity is associated with serious pathological consequences of T2DM mellitus and cardiovascular disease (Flier, 2004).

Although, progression of T2DM occurs more frequently in obese humans and rodents as compared to lean individuals, it is also highly dependent on genetic factors. Genetic susceptibility increases the risk of T2DM in obese states. Likewise, many obese humans do not progress to T2DM due to reduced genetic risk. This suggests that genetic and environmental factors also play a very significant role in pathogenesis of T2DM mellitus. Therefore, there is a general understanding that two factors are critical for obesity to progress to T2DM. First, impaired insulin insensitivity in skeletal muscles and second, a precondition for the onset of T2DM.

Insulin resistant individuals that are not diabetic, are hyperinsulinimic as their β-cells start to synthesize more insulin to compensate for its resistance in skeletal muscle. For insulin resistance to develop into T2DM failure of β-cells to synthesize enough insulin to keep fasting plasma glucose levels under control is a prerequisite (Butler *et al.*, 2003; Kahn, 2003; Rhodes, 2005).

2.2.1 Adipocytes and Insulin Resistance

Adipose tissues have two main specialized functions: sequestration of fats into triglycerides in order to store excess energy leading to reduction in levels of circulatory FFAs which have many deleterious effects on metabolic homeostasis (Unger, 1995) and secretion of appropriate levels of adipokines which affect whole-body homeostasis and neuroendocrine regulation of patterns of food intake (Ahima and Flier, 2000; Berg *et al.*, 2002). Importance of adipose tissue in regulating metabolic homeostasis by fat sequestration is reinforced by the observation that lack of adipose tissue correlates with elevated levels of circulatory FFAs which subsequently lead to insulin resistance in humans and mice (Shimomura *et al.*,

1998; Sovik *et al.*, 1996). Adipocytes release energy in the form of free fatty acids by breakdown of stored triglycerides during the process of lipolysis. FFAs have been implicated in mediating insulin resistance in studies which show strong association of obesity and insulin resistance with elevated FFAs (Savage *et al.*, 2007) (Figure.2.3). Furthermore, FFAs have also been shown to cause peripheral insulin resistance in both humans and animals (Boden, 1997; Kelley *et al.*, 1993). Administration of antilipolytic drug acipimox shows increase in insulin-stimulated glucose uptake in the periphery (Santomauro *et al.*, 1999).

2.2.2 Adipose Tissue Dysfunction in Obesity

In fasting state, a dynamic equilibrium exists between the release of FFAs into circulation and their uptake by peripheral tissues mainly the skeletal muscle (Rosen and Spiegelman, 2006). Studies on humans and rodents with high caloric intake have shown that any change in adipose tissue alters the equilibrium between fatty acid release and use. In normal lean states, when FFAs are picked up from the circulatory system by cells of peripheral tissues, they are esterified with co-enzyme A to reduce their toxicity and detergent properties. In the lean state, levels of fatty acyl-CoA are less owing to their rapid oxidation by mitochondria.

In hyperphagia-induced obesity, increased caloric intake leads to enlargement of adipocytes due to high deposition levels of triglycerides. At early stages, adipocytes continue to store triglycerides and maintain normal rates of lipolysis (Guilherme *et al.*, 2008).

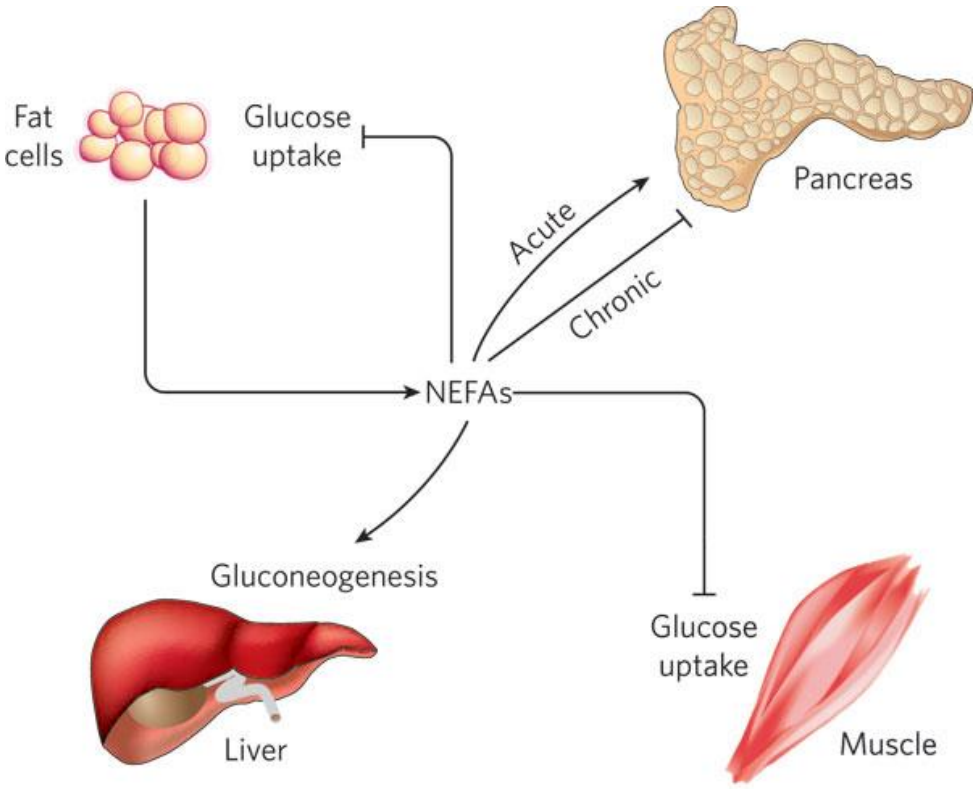

Figure 2.2. Effect of adipocyte-derived non-esterified fatty acids (NEFAs) on glucose homeostasis. Insulin represses lipolysis in adipocytes, therefore, when insulin levels are low in fasting state lipolysis occurs without any hindrance. Insulin resistance, however, results in inhibition of the antilipolytic effect causing release of NEFA into the circulation. Elevated serum NEFAs prevent peripheral glucose uptake into muscle and fat and enhance hepatic glucose production (Rosen and Spiegelman, 2006)

As adiposity increases, the ability of adipocytes to function as a site of synthesis of adipokines and other proteins that regulate metabolism is highly compromised. This dysfunction of adipocytes arises from irregularities of triglyceride and fatty acid metabolism in adipocytes themselves. Adipokines that are released from adipocytes regulate not only the whole-body metabolism but also the metabolism of adipocytes as well. Examples of such adipokines are monocyte chemoattractant protein-1/chemokine (C–C motif) ligand-2 (MCP-1/CCL2) and tumor-necrosis factor-α (TNFα) where the latter modulates an inflammatory response in adipose tissue (Sartipy and Loskutoff, 2003). As a result of this inflammatory response, macrophages infiltrate the adipose tissue in obese mice (Xu *et al.,* 2003b) and humans (Curat *et al.,* 2004). Inflammatory response mediated by adipokines regulates adipocyte metabolism and their ability to store triglycerides.

Adipose tissue inflammation in obesity is associated with insulin resistance in skeletal muscle. During this state, adipocytes and macrophages secrete MCP-1 and other chemoattractants for inflammatory cells as well as large amounts of TNFα, interleukin-1 β (IL-1β) and other inflammatory cytokines (Lagathu *et al.*, 2006). These inflammatory cytokines have two drastic effects on adipocyte function: increase in lipolysis and reduction in triglyceride synthesis. This leads to an overall increase in release of circulatory FFAs.

2.2.3 Adipokine Synthesis

Adipose tissue secretes a wide variety of proteins and hormones referred to as adipokines which act as feedback signals to regulate adipocyte metabolism (Table 2.1).

2.2.3.1 Leptin

Leptin (*LEP*) or obese (*ob*) gene was the first adipokine to be identified by Friedman and colleagues (Zhang *et al.*, 1994b). Leptin has been described as an adipokine which regulates food intake and energy expenditure as well as reproductive, immune and other endocrine system (Moschos *et al.*, 2002). Leptin injection into normal and *ob/ob* mice (two to three times obese than the normal ones) reduces food intake largely, increased energy expenditure by enhanced physical activity and thermogenesis (Moschos *et al.*, 2002; Zhang *et al.*, 1994a). In the light of its actions, leptin is known as an 'adipostat' which triggers the brain to suppress food intake and increase energy expenditure (Bjorbaek and Kahn, 2004; Rosen and Spiegelman, 2006). Insulin has been shown to stimulate secretion of leptin whereas leptin suppresses insulin secretion in a negative-feedback loop (Barr *et al.*, 1997). Several studies have shown that acute administration of leptin in hyperinsulinimic or fasting mice, improves glucose metabolism and insulin sensitivity (Kamohara *et al.*, 1997) and prolonged treatment can also reverse insulin resistance in mice with congenital lipodystrophy (Shimomura *et al.*, 1999). Leptin therapy in humans with lipodystrophy and insulin resistance improves insulin action and sensitivity and also reduces lipid content in liver and muscle (Petersen *et al.*, 2002).

2.2.3.2 Adipsin

Adipsin, first described in 1987 (Cook *et al.*, 1987) is one of the major proteins produced by adipocytes. It is a serine protease which catalyzes the rate-limiting step of the alternative pathway of complement activation. Later, adipsin was also found to be a homolog of complement factor D (CFD) in humans (Rosen *et al.*, 1989). Unlike rodents, in humans

adipsin/CFD is also secrete by monocytes/macrophages into the bloodstream besides adipocytes (White *et al.*, 1992). Levels of adipsin have been found to decrease in animal models of obesity and diabetes (Flier *et al.*, 1987). As human patients with T2D eventually develop beta-cell failure (Ferrannini, 2014), a recent study has reported that expression of adipsin is significantly downregulated in T2DM patients with beta-cell failure as compared to T2DM patients without beta-cell failure. The results suggested that adipsin has a strong correlation with beta-cell function which was improved when *db/db* mice were given adipsin therapy resulting in enhanced glucose-stimulated insulin secretion (Lo *et al.*, 2014).

2.2.3.3 Adiponectin

Adiponectin is the only highly expressed adipokine in the bloodstream. It is encoded by the ADIPOQ gene and regulates signaling in its target cells via AdipoR1 and AdipoR2 receptors (Kadowaki *et al.*, 2006; Yamauchi *et al.*, 2003). Studies in rodents have revealed Adiponectin to possess anti-inflammatory, anti-atherogenic and insulin sensitizing properties (Bell-Anderson, 2008; Yamauchi *et al.*, 2001). Levels of adiponectin in circulation have been found to be closely correlated with incidence of insulin resistance, T2DM and dyslipidemia (Mohan *et al.*, 2005). Adiponectin stimulates insulin sensitivity by enhancing expression of proteins which increase fatty acid oxidation and mobilization of triglycerides stored in muscle and liver in obese mice (Fruebis *et al.*, 2001; Xu *et al.*, 2003a). Adiponectin and inflammatory cytokines such as TNFα and IL-6β are reported to function in a negative feedback loop. High caloric intake or over nutrition results in an increase in inflammatory cytokines which eventually downregulate the expression and secretion of adiponectin thus increasing susceptibility to developing insulin resistance (Bruun *et al.*, 2003; Hector *et al.*, 2007).

2.2.3.4 Resistin

Resistin is a polypeptide synthesized by adipocytes playing an important role in development of insulin resistance. It was during an attempt to search for adipose-tissue specific proteins downregulated in response to thiazolidinedione (TZD), an antidiabetic drug, that resistin was discovered (Steppan *et al.,* 2001a). It is a cysteine rich protein which belongs to the family of resistin-like molecules also known as 'found in inflammatory zone' (FIZZ) (Steppan *et al.,* 2001b). Levels of resistin are elevated in hyperglycemic conditions in many murine models of obesity (Banerjee *et al.,* 2004). Resistin has been named so due to its ability of causing insulin resistance. Administration of anti-resistin antibodies to obese and insulin resistant mice improves insulin sensitivity and glucose tolerance (McTernan *et al.*, 2002). Moreover, down regulation of resistin upon treatment with antidiabetic drugs like thiazolidinedione and rosiglitazone, further implicate resistin to be a culprit in development of insulin resistance and hence, T2DM (McTernan *et al.*, 2002; Steppan *et al.*, 2001a).

2.2.3.5 Tumor Necrosis Factor- α

Tumor necrosis factor-α was the first adipokine shown to affect glucose homeostasis (Hotamisligil, 1999). Obesity and insulin resistant states are characterized by elevated TNF-α levels. Moreover, addition of TNF-α to cells and mice reduces insulin action, and inhibition of TNF-α restores insulin sensitivity *in vivo* and *in vitro*. TNF-α is largely synthesized by macrophages that have infiltrated the adipose tissue due to inflammatory reaction (Xu *et al.,* 2003c). Adipokines like TNF-α can promote insulin resistance through several mechanisms including c-Jun N-terminal kinase 1 (JNK1)-mediated serine

phosphorylation of insulin receptor substrate-1 (IRS-1) (Hirosumi *et al.*, 2002), IκB kinase (IKK)-mediated nuclear factor-κB (NF-κB) activation (Shoelson *et al.*, 2003), induction of suppressor of cytokine signaling 3 (SOCS3) (Howard and Flier, 2006) and production of ROS (Houstis *et al.*, 2006).

2.2.4 Role of TNFα in Adipose Tissue Inflammation

There is evidence from numerous studies that supports the observation that levels of TNFα are profoundly elevated in inflamed adipose tissue. Tumor Necrosis Factor α has been found to affect adipocytes severely and compromise their ability to function efficiently. Tumor Necrosis Factor α attenuates insulin signaling and adipogenesis thereby affecting the ability of adipocytes to store triglycerides and synthesize adipokines.

PPARγ is a nuclear hormone receptor which acts a key transcriptional factor of adipogenesis and maintenance of adipocyte function (Imai *et al.*, 2004; Tamori *et al.*, 2002). Evidence suggests that increase in levels of TNFα subsequently downregulates PPARγ and many other adipocyte specific genes (Stephens *et al.*, 1997; Zhang *et al.*, 1996). TNFα brings about downregulation of PPARγ by activating NF-κB and AP-1 transcription factors via NF-κB and MAP Kinase pathway (Ruan *et al.*, 2002).

In adipocytes, rate of lipolysis is regulated by the hormone insulin via cAMP. Cyclic AMP regulates the Protein Kinase A (PKA)-dependent phosphorylation of hormone-sensitive lipase (HSL) (Granneman and Moore, 2008; Large *et al.*, 2004). Phosphorylated and activated HSL goes on to hydrolyze triglycerides in fat droplets of adipocytes thereby releasing glycerol and free fatty acids. Besides HSL, another enzyme, adipose triglyceride lipase (ATGL) also contributes in hydrolysis of triglycerides.

Table 2.1 Adipokines and their associated metabolic function

Adipokine	Metabolic Function	Reference
Adiponectin	Improves glucose-stimulated insulin secretion, improves glycemic control and glucose tolerance, anti-inflammatory, anti-atherogenic	(Hu *et al.*, 1996; Miller *et al.*, 2011; Wijesekara *et al.*, 2010)
Adipsin	Enhances insulin secretion from β-cells by improving their function, downregulated expression in obesity and type 2 diabetes	(Choy *et al.*, 1992)
Leptin	Regulates appetite and food intake, regulates energy expenditure, improves insulin sensitivity, prevents increase in adipose tissue mass	(Frühbeck *et al.*, 1998; Shimomura *et al.*, 1999)
Resistin	Causes insulin insensitivity in obese and Type 2 Diabetic individuals, increased expression in T2DM	(Shimomura *et al.*, 1999)

However, research suggests that ATGL is not important for catecholamine-stimulated lipolysis that occurs during fasting (Ryden *et al.*, 2007). In fed-state, activation of insulin receptor in adipocytes results in activation of PI(3)K signaling pathway resulting in a consequent decrease in intercellular cAMP. Reduction in cAMP means reduction in the rate of lipolysis. Conversely, in fasting state, levels of cAMP increase by activation of adenyl cyclase by adenoreceptors (Degerman *et al.*, 2001; Granneman and Moore, 2008).

In the light of evidences from various researches, TNFα appears to have a strong correlation with adipocyte inflammation and enhanced lipolysis. Due to inflammation, TNFα is secreted by adipocytes and macrophages within the adipose tissue of obese humans and animals (Wellen and Hotamisligil, 2003) and stimulates lipolysis (Langin and Arner, 2006), attenuates insulin signaling thereby causing insulin resistance (Hotamisligil *et al.*, 1995). Increase of lipolysis has been proposed to occur by three main mechanisms: attenuation of the antilipolytic effect of insulin by reducing insulin signaling; enhancement of cAMP levels; and suppression of perilipin function (Langin and Arner, 2006). TNFα induced downregulation and suppression of perilipin facilitates association of HSL with triglycerides in fat droplets of adipocytes resulting in release of fatty acids into the circulatory system (Figure 2.4).

2.3 Epigenetic Regulation in Type 2 Diabetes

Epigenetics is defined as the study of DNA methylations and histone modification which act as an inheritable and reversible phenomenon regulating gene expression without altering the primary DNA sequence (Keating and El-Osta, 2012). Epigenomics is defined as the study of genome-wide epigenetic modification. Epigenetics is used to study the link

between genes and environment during disease development. It has proven to be a useful technique to study genetic fetal programming, differences between monozygotic twins and onset of chronic diseases in adults in light of their diet and nutrition patterns. DNA methylation has been one of the widely studied epigenetic modification as compared to other studies of histone modification. Epigenetic modifications and other regulatory processes like transcriptional regulation form a regulatory mechanism which controls gene activity and expression during development and differentiation or in response to nutritional or environmental stimuli.

2.3.1 Histone Modifications

Genomic DNA in eukaryotes is packaged with histone proteins which compact the DNA by 10,000 folds. This degree of compaction limits the access of the nuclear machinery to the DNA for processes like replication, transcription and DNA repair. This problem is overcome by the dynamic changes in the degree of DNA compaction owing to changes in post-translational modifications of histones. The basic repeating unit of chromatin is a nucleosome which is composed of an octamer of four histone proteins, H2A, H2B, H3 and H4 around which 146 base pairs of DNA is wrapped (Luger *et al.,* 1997). Each core histone protein is composed of a structured domain and an unstructured amino-terminal tail comprising of 25-40 residues. These histone tails protrude out of the nucleosome into the space between adjacent nucleosomes and undergoes various histone modifications including acetylation, methylation and phosphorylation.

Histone acetylation occurring at the Ɛ-amino group of lysine residues in the amino-terminal tails has long been associated with activation of gene transcription (Allfrey *et al.,* 1964).

Addition of acetyl groups to lysine residues neutralizes the overall charge of histones thereby reducing their affinity for DNA and alters interactions with other histone and regulatory proteins (Grant and Berger, 1999). Histone acetylation renders the chromatin transcriptionally active whereas deacetylation makes the chromatin transcriptionally inactive (Roth *et al.*, 2001). Addition of acetyl groups is mediated by histone acetyltransferases (HATs) whereas deacetylation is carried out by Histone Deacetylases (HDACs).

Histone phosphorylation occurs on serine, threonine and tyrosine residues predominantly in the amino-terminal tail but also in the structured domain (Xhemalce *et al.*, 2006). Phosphorylation of histones is mediated by kinases and phosphatases which add and remove a phosphate group, respectively (Oki *et al.*, 2007). Addition of phosphate groups, adds substantial negative charge to the protein affecting the chromatin structure. All of the kinases identified so far, transfer the phosphate group from ATP to the hydroxyl group of the target site (Bannister and Kouzarides, 2011).

Histone methylation occurs mainly on the side chains of lysine and arginine residues. However, unlike acetylation and phosphorylation, methylation does not alter the overall charge of the histone proteins. Lysine residues can be mono-, di-, or tri-methylated and arginines can be mono-, symmetrically or asymmetrically methylated (Ng *et al.*, 2009) . Lysine and arginine methylation is mediated by lysine methyltransferases and arginine methyltransferases respectively.

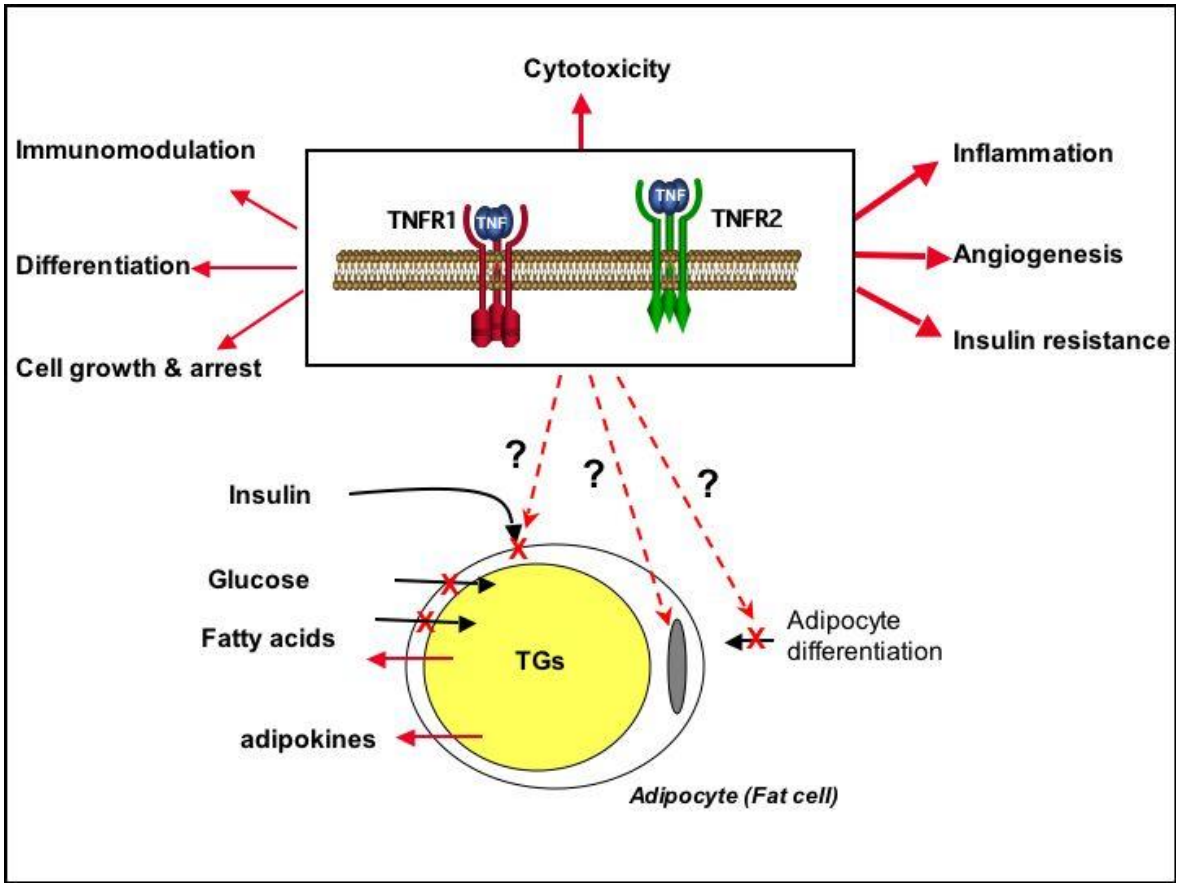

Figure 2.3 TNF alpha and its various functions. TNF alpha (or Tumor Necrosis Factor alpha) is a multifunctional cytokine that mediates its effects via two cell surface receptors. These are ubiquitously expressed and primarily differ in their intracellular signaling pathways. Both TNF and its receptors are produced by adipose tissue where it can affect normal adipocyte development as well as key functions such as insulin sensitivity, lipid metabolism and adipokine production. These actions can themselves go on to alter the whole body energy homeostasis (http://www.clbc.cam.ac.uk/Sethi/Sethi_Lab.htm).

2.3.2 DNA Methylation

DNA methylation refers to the addition of methyl groups to C5 of cytosine residues to form 5-methylcytosine primarily in the CpG sites within the primary genomic sequence. DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that transfer a methyl group from *S*-adenyl methionine (SAM) to a cytosine residue. Cytosine methylation of DNA leads to transcriptional repression of the methylated region as it hinders the association of transcription factors with DNA (Iguchi-Ariga and Schaffner, 1989). Methylated CpG residues are recognized and bound by methyl-CpG binding proteins which are associated with chromatin modifiers to bring about transcriptional repression changes in chromatin (Fuks *et al.*, 2003; Zhang *et al.*, 1999). Dnmt1 is involved in methylation of the DNA daughter strand produced during replication to generate the exact methylation pattern as that of the parent strand. Dnmt2 and Dnmt3 are capable of establishing a new DNA methylation pattern on unmodified DNA and are therefore known as *de novo* Dnmt. As cells reach terminal differentiation, Dnmt expression is reduced suggesting that DNA methylation pattern remains stable after cell mitosis However, Dnmt expression in postmitotic neurons has been shown to be significant suggesting the possibility of a novel role DNA methylation and Dnmts in brain (Goto *et al.*, 1994). DNA methylation plays a critical role in regulating tissue-specific gene expression, inactivation of X-chromosome, and silencing of retroviral elements.

2.3.2.1 DNA Methylation of Intergenic Regions

Approximately 45% of the mammalian genome consists of transposable elements and viral elements, which if not silenced, replicate and get inserted into different regions of genome

giving rise to harmful mutations and gene disruption (Gwynn *et al.*, 1998; Ukai *et al.*, 2003). To prevent this scenario, these intergenic regions are silenced by either bulk DNA methylation (Schulz *et al.*, 2006) or by mutations acquired overtime by deamination of 5mCytosine (Walsh *et al.*).

2.3.2.2 DNA Methylation of CpG Islands

CpG islands are 1000 base pair long stretches of DNA rich in CpG sites as compared to the rest of genome but are often not methylated (Bird *et al.*, 1985). Roughly 70% of gene promoters reside within CpG islands (Saxonov *et al.*, 2006). CpG islands which are associated with promoter regions are highly conserved between humans and mice (Illingworth *et al.*, 2010). This perseverance of CpG islands across species during the course of evolution suggests a critical role CpG islands play in gene regulation.

CpG islands have been evolutionary conserved to regulate chromatin structure and gene expression. One of the common features shared by different CpG islands is that they contain less number of nucleosomes (Choi, 2010; Ramirez-Carrozzi *et al.*, 2009) as compared to the rest of genome. Moreover, nucleosomes at CpG islands contain less amount of histone H1 and have highly acetylated H3 and H4 promoting gene transcription (Tazi and Bird, 1990). As gene promoter regions are predominantly associated with CpG islands, histone modifications promoting gene expression as well as the fact that most transcription factors recognize GC rich regions for binding allow for a general conclusion that CpG islands are designed to promote gene expression by making DNA accessible to transcription factors.

Methylation of DNA results in stable gene silencing (Mohn *et al.*, 2008). During early embryonic development and gametogenesis, CpG islands undergo differential methylation (Wutz *et al.*, 1997; Zwart *et al.*, 2001). DNA methylation plays a significant role in gene imprinting where only one of the two genes from both parental chromosomes is expressed (Zwart *et al.*, 2001). Gene expression during development and differentiation is also regulated by DNA methylation (Mohn *et al.*, 2008; Shen *et al.*, 2007). Owing to its function of regulating gene expression, DNA methylation regulates tissue-specific expression of gene body and intragenic regions, however, methylation patterns of transcription start sites are not often tissue specific (Illingworth *et al.*, 2010; Rakyan *et al.*, 2004).

2.3.2.3 DNA Methylation of Gene Body

Gene body is the region of the gene past the first exon. Methylation of the first exon, just like the promoter, leads to transcriptional repression (Brenet *et al.*, 2011). Evidence has shown that genes which are highly expressed exhibit low promoter methylation and high methylation of the gene body (Aran *et al.*, 2011; Ball *et al.*, 2009). This pattern of gene-body hypermethylation does not only persist in active gene of proliferating cells but also in tissues containing few proliferative cells (Aran *et al.*, 2011).

2.3.3 Epigenetic Changes and Diabetes

There have been but a few studies focusing on the role of DNA methylation in pathogenesis of diabetes, however, there have been evidence of altered methylation patterns in patients with diabetes (Maier and Olek, 2002). Research has shown that exposure to poor nutrition in intrauterine environment might predispose the fetus to developing diabetes later in life owing to aberrant DNA methylation patterns in pancreatic islets of rats (Thompson *et al.*,

2010) and at the HNF4A gene locus of CD34+ stem cells from cord blood of neonates (Einstein *et al.,* 2010). Most importantly, insulin expression has also been shown to be regulated by DNA methylation. The INS promoter has been found hypermethylated in mouse embryonic stem cells. However, the promoter gets demethylated when stem cells differentiate into β-cells specialized for insulin secretion. This finding has also been extended and confirmed in human insulin gene (Kuroda *et al.,* 2009).

Recent studies have explored the relationship between histone hyperacetylation and gene expression under diabetic conditions. Cultured primary human endothelial cells under hyperglycemic conditions displayed increased expression of p300 acetyltransferase and enrichment of this enzyme to the promoters of HG-responsive genes such as fibronectin (Chen *et al.,* 2010). Hyperacetylation of histone H4 at the promoter enhances expression of the INS gene (Mosley and Özcan, 2003). Furthermore, the β-cell specific transcription factor PDX1 has been shown to interact with p300 to mediate proinsulin gene expression (Mosley *et al.,* 2004). Thus, histone acetylation is involved in both regulation of insulin secretion and hyperglycaemia associated damage. Therapeutic targeting of the enzymes that regulate these processes may provide a potential therapy to alleviate some aspects of the disease.

MATERIALS AND METHODS

3.1 Study Subjects

This was a case control study which compared the epigenetic changes of T2DM patients with those of non-diabetes controls. A total of 21 human subjects were recruited in this study. T2DM patients with and without insulin treatment were included in the study while those with other associated diseases e.g. cardiac disorders, hypertension were disqualified from the study population. 10 subjects were categorized as controls, 4 as patients with T2DM on insulin treatment and 6 as patients with T2DM without insulin treatment. Thus, the samples were categorized into three groups:

1) Control group: Non-diabetics
2) Type 2 Diabetics on insulin treatment
3) Type 2 Diabetics without insulin treatment

3.1.2 Sample Collection and Storage

Subcutaneous adipose tissue samples were collected from patients undergoing elective surgeries like cholecystectomy and inguinal hernia surgery at Holy Family Hospital, Rawalpindi and Bilal Hospital, Rawalpindi. The samples were collected with complete consent from patients. Tissue samples weighing 5g to 7g were collected in 15ml sterilized falcons and immediately snap-frozen in liquid nitrogen while transportation and later stored at -80 degree Celsius for long-term storage.

3.2 Primer Designing

Gene sequences of β-Actin which acts as an internal control and TNFα were retrieved from NCBI Gene Bank and their primers were designed using Primer3 (http://primer3.ut.ee/) and Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 3.1). Other properties like complementarity were evaluated using Oligocalc, an online oligonucleotide properties calculator ((http://www.basic.northwestern.edu/biotools/oligocalc.html). The primers were also subjected to In Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) to check for their specificity and efficiency.

3.3. RNA Isolation from Adipose Tissue

Cellular RNA was extracted using TRIzol® reagent (Catalog No.15596-026, Invitrogen). Homogenization of sample, phase separation, RNA precipitation, washing and solubilization constitute the main stages of RNA extraction.

A mortar and pestle was used to homogenize 50 to 100mg of tissue sample. Liquid nitrogen was used to grind the tissue to obtain a fine powder. Care was taken not to allow thawing of tissue at room temperature as it leads to RNA degradation. Powdered tissue sample was transferred to 1ml of Trizol in a 1.5ml Eppendorf tube and vortexed for a few seconds to allow for thorough homogenization. The homogenate was then pre-cleared for 10 minutes at 12000g at 4 degree Celsius in a refrigerated centrifuge in order to separate tissue debris and fats which form the supernatant. The intermediate red/pink interphase was transferred to a new microcentrifuge tube and left for 5 min at room temperature. 20µL of freshly prepared 5N Glacial Acetic Acid was then added to the microcentrifuge tube and mixed

vigorously 15 seconds followed by a 5 minute incubation at room temperature. For phase separation, 200µL of chloroform was added to the homogenate and mixed vigorously for 15 seconds as shaking results in better emulsification and a higher volume of aqueous phase later. Following shaking with chloroform, the homogenized sample was incubated at room temperature for 10 minutes and then centrifuged at 12000 x g for 15 minutes at 4°C. This centrifugation resulted in phase separation: upper clear aqueous phase containing RNA, white interphase containing DNA and the lower pink phenol-chlorofom phase containing proteins. The clear aqueous phase containing RNA was aspirated carefully with a micropipette and transferred to a fresh tube. 500µL of chilled Isopropanol was added to the aqueous layer to allow for RNA precipitation. RNA being insoluble in isopropanol, aggregates together forming a pellet upon centrifugation. The aqueous phase with Isopropanol was incubated at room temperature for 5-10 minutes and then centrifuged at 12000 x g for 10 minutes at 4°C. The resulting supernatant was discarded and the precipitated RNA was washed with 1ml of 75% ethanol in DEPC-treated water and vortexed for a few seconds followed by a centrifugation at 7500 rcf for 5 minutes at 4°C. The supernatant was decanted and the pellet was air dried in a laminar flow hood for approximately 5 minutes. RNA pellet was re-suspended in 25µL of DEPC-treated water and incubated for 10-15 minutes at 55°C to 60°C.

3.3.1 RNA Quantification

The quality and quantity of extracted RNA from tissue samples was analyzed by using Thermo Scientific Nanodrop 2000 UV-Vis Spectrophotometer and NanoDrop™ 2000 software at KRL Hospital, Islamabad. RNA was quantified at a wavelength of 260nm as this wavelength provides the absorption maxima for nucleic acids. Ratio of absorbance at

260nm and 280nm i.e. $A_{260/280}$ is used to determine the purity of the product. Absorbance ratio of 1.8 and 1.9 indicates up to 80% weight of protein contamination. 1µL of RNA suspended in DEPC treated water was used for quantification, while pure DEPC treated water was used a blank for accuracy of quantification.

3.3.2 cDNA Synthesis

Complementary DNA was synthesized from extracted RNA using $Oligo(dT)_{18}$ (Life Technologies #SO131) primer, RevertAid Reverse Transcriptase (Thermo Scientific #EP0442) and RiboLock RNase Inhibitor (Thermo Scientific #EO0381). $Oligo(dT)_{18}$ primer is a synthetic single stranded 18-mer oligonucleotide which recognizes PolyA tails of mRNA initiating reverse transcription of RNA into cDNA. For one reaction, 1µ of template RNA, 0.5µg $Oligo(dT)_{18}$ and DEPC-treated water was used to raise the reaction volume up to 12.5µL.

Contents of the tube were gently mixed, centrifuged briefly and incubated at 65°C for 5 minutes and then quickly chilled on ice. The tube was once again centrifuged briefly and placed on ice for the next step. Remaining components were then added including 4 µL of 5X reaction buffer, 20U of RiboLock RNase Inhibitor, 10mM dNTP mix, 200U of RevertAid Reverse Transcriptase and the total volume was raised up to 20 µL.

The reaction tube was incubated for 60 minutes at 42°C which is the optimal temperature for reverse transcriptase enzyme. After 60 minutes, the reaction was terminated by heating at 70°C for 10 minutes. The cDNA reaction products were stored at -20°C for later use in RCR and real-time PCR.

All the microcentrifuge tubes, pipette tips used in RNA isolation and cDNA synthesis were either certified RNase free by manufacturer or were made RNase free by treating with 1% DEPC treated water and were autoclaved before use. cDNA synthesis was confirmed by using it as a template for amplification of β-globin which is used as a housekeeping gene. The amplification was done on a conventional PCR using a protocol discussed in coming sections.

3.4 Semi-quantitative PCR

For both TNFα and β-Actin, PCR profile and amplification conditions were first optimized on a conventional PCR (BioRad). A total amount of 25µg of cDNA was used in each reaction. The primers had a working stock of 10µM and were added into the reaction mixture at a concentration of 0.5 µM. The reaction mixture for each gene primer was prepared by adding 2µL of 10X Taq Buffer with (NH4)2SO4, 25mM of $MgCl_2$, 2mM dNTPs Mix, 0.5 µM of each forward and reverse primers, 25 ng of template cDNA, 2.5U Taq Polymerase and Nuclease free water was used to raise the total reaction volume up to 20 µL.

The reaction mixture was next centrifuged briefly for 5 seconds for thorough mixing and any air bubbles formed during pipetting were also tapped out. Amplification profile consisted of initial denaturation at 95°C for 5 minutes, 35 repeated cycles of denaturation at 95°C for 40 seconds, amplification at 60°C for 40 seconds, annealing at 72°C for 40 seconds and a final extension at 72°C for 10 minutes (Figure 3.1). In order to confirm amplification of the desired genes, the PCR products were mixed with 3 µL of loading dye (0.25% Bromophenol blue in 40% sucrose solution) and loaded into the wells of a 2% agarose gel. The agarose gel was made by dissolving 1g of agarose in 50 ml of 1X TAE

Table 3.1 Primer Sequences for expression analysis

S.No	Primer	Sequence	Product Size
1.	TNFα-Forward	ATGAGCACTGAAAGCATGATCC	217 bp
2.	TNFα-Reverse	GAGGGCTGATTAGAGAGAGGTC	
3.	B-actin-Forward	GGACTTCGAGCAAGAGATGG	229 bp
4.	B-actin-Reverse	TGTGTTGGCGTACAGGTCTTTG	

3.4.1 Real-time PCR

After optimizing the primers and reaction conditions on the conventional PCR, the amplification conditions were tested on Applied Biosystems 7300 Real-Time PCR System by using Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Catalog No: K0221, Fermentas).

The reaction mixture for real time PCR was prepared by using 12.5 µL of 2X qPCR Master mix, 0.5 µM of each forward and reverse primer, 25 ng of template cDNA and reaction volume was raised up to 25 µL by Nuclease-free water. The reactions profile consisted of an optional UDG pre-treatment at 50 ºC for two minutes followed by an initial denaturation at 95 ºC for 10 minutes. There were then 40 repeated cycles of amplification at 95 ºC (denaturation) for 15 seconds, 60 ºC (annealing) for 30 seconds and extension at 72 ºC for 30 seconds. One cycle of dissociation stage was included with a profile of denaturation at 95 ºC for 15 seconds, 60 ºC for 30 seconds and 95 ºC for 15 seconds. The dissociation stage was carried out for melt-curve analysis which helps in identifying presence of primer dimers or any non-specific amplification. Data acquisition was performed during the annealing/extension step.

The first step was to optimize the primers and amplification conditions on real-time PCR too. The optimization also followed the same recipe as described above but after the reaction completion included an additional step of running the products on 2% agarose gel made in 1X TAE buffer. 10µL PCR products were mixed in 5µL 6X loading dye and loaded into the wells along with 50bp DNA ladder (Thermo Scientific, EU, Lithuania) in an electrophoresis tank. Gel stained with Ethidium bromide was visualized in Wealtec

Dolphin Doc (S/N470883) gel documentation system and specific bands were observed for respective product sizes.

3.4.2 Data Analysis

Ct values of the target genes were compared to the reference housekeeping gene β-actin and fold change in the expression of target gene between control and experimental groups was calculated as:

Ct Value of β-actin in Control = X1

Ct Value of TNFα in Control = X2

Ct Value of β-actin in Experimental = Y1

Ct Value of TNFα in Experimental = Y2

ΔCt Control = (Ct of TNFα in Control) - (Ct of β-actin in Control)

$= X2-X1= \Delta Ct_{control}$

ΔCt Exp = (Ct of TNFα in Exp.) - (Ct of β-actin in Exp.)

$= Y2- Y1= \Delta Ct_{experimental}$

Calculate $\mathbf{2^{-\Delta Ct}}$ for each experimental and control groups.

To calculate Fold Change between experimental and control groups:

Fold Change = $2^{-\Delta Ct}$ for Experimental / $2^{-\Delta Ct}$ for Control

3.5 DNA Methylation of TNFα Promoter

For analysis of DNA methylation, whole DNA was extracted from adipose tissues and then subjected to Bisulfite conversion using the Bisulfite conversion kit. The converted DNA was subsequently used in methylation specific PCR (MSP). For MSP, two primer pairs

were used each against a different loci on the TNFα promoter. For each of the two loci, a methylation specific and a non-methylation specific primer was used in order to account for both methylation and unmethylation at the particular site.

3.5.1 DNA Extraction from Adipose Tissues:

First step in DNA extraction was homogenization of 2-3 grams of tissue sample using liquid nitrogen ($N_{2\ (l)}$) which was carried out using a mortar and pestle. The tissue was grinded until a fine powder was obtained. For cell lysis, lysis saline buffer was prepared by adding 20µL of 1M Tris-HCl; pH 8.0, 50µL of 1M NaCl, 50µL of 10% SDS, 50µL of 1M EDTA solution; pH 8.0 and biology grade water. In a sterilized eppendorf tube homogenized tissue was suspended in lysis buffer, 12.5µL of 20 mg/mL proteinase K and biology grade water (to make 500 µL in total). Tissue homogenate in lysis buffer was incubated overnight at 55^0C.

Next day, the homogenate was incubated in a shaking incubator at 55^0C for half an hour. After incubation, 200µL of 6M NaCl was added into the same tube to allow for cell lysis which was aided by vigorous shaking. The tissue homogenate was centrifuged at 14000 rpm at room temperature. The resulting supernatant was transferred to a new tube with the help of a micropipette. After that, 500µL of isopropanol was added to the supernatant and vortexed for a few seconds followed by and centrifugation at 14000 rpm. After precipitation, DNA formed a white pellet at the base of the tube. Supernatant was discarded and the DNA pellet was air dried. In the next step, DNA was washed with 1ml of 70% ethanol followed by centrifuged at 14000 rpm. Supernatant was decanted and pellet was incubated at 55^0C to dry it. The washed and dried DNA pellet was re-suspended in 20µL

TE buffer (10mM Tris–HCl, 0.5mM EDTA, pH 7.6) and dissolved by pipetting up and down. It was incubated in shaking incubator at 37^0C for one and a half hour to completely dissolve DNA in TE buffer.

3.5.2 DNA Quantification:

Genomic DNA was quantified using ThermoScientific Nanodrop 2000 UV-Vis Spectrophotometer and NanoDrop™ 2000 software at KRL Hospital, Islamabad. Absorbance wavelength of 260nm was selected as this provides the maximum absorption for nucleic acids. TE buffer was used as a blank since DNA was re-suspended in it after extraction from the tissue. Absorbance ratio i.e. $A_{260/280}$ was determined to check for level of DNA purity.

3.5.3 Bisulfite Conversion:

For DNA methylation analysis, DNA was subjected to bisulfite conversion using Thermo Scientific EpiJET Bisulfite Conversion Kit (Cat #K1461). In the bisulfite reaction all unmethylated cytosines are deaminated and converted to uracils, while methylated cytosines remain unchanged. Bisulfite converted unmethylated cytosines are detected as thymine after the PCR reaction.

3.5.3.1 Reagent Preparation:

Reagents used in Bisulfite conversion of DNA were supplied by the manufacturer in the Bisulfite conversion kit as dry mixtures or concentrated form which then needed to be dissolved or diluted before use.

- **Modification Reagent:**

Modification Reagent was a dry mixture and needed to be dissolved before use. The amount of Modification Reagent in each vial given was sufficient for 10 DNA conversion reactions. Each vial was therefore prepared by the addition of 0.9 mL of molecular biology grade water, 200 µL of Modification Solution I and 60 µL of Modification Solution II. The contents were then dissolved by inverting for about 10 minutes.

- **Wash Buffer:**

Wash buffer was provided in concentrated form and prepared by diluting it with 25 ml of 96-100% ethanol.

- **Desulfonation Buffer:**

Desulfonation buffer was also supplied in a concentrated form and was prepared for use by diluting it with 10 mL of 96-100% ethanol.

3.5.4 Bisulfite Conversion of DNA:

In a PCR tube, for a reaction of 20 µL, 500 ng of purified genomic DNA was added and if the volume was less than 20 µL, it was raised to 20 µL by adding nuclease free water. 120 µL of prepared Modification Reagent was then added to the DNA and mixed by pipetting up and down followed by a brief centrifugation. The PCR tubes were then placed in a thermal cycler and heated at 98 degrees Celsius for 10 minutes and at 60 degrees Celsius

for 150 minutes. The resulting product (converted DNA) was added to 400 µL of Binding Buffer in a DNA Purification Micro Column placed in a collection tube. The micro column placed into the collection tube was centrifuged at 12,000 rpm for 30 seconds and flow-through was discarded. The Micro Column was placed into the same collection tube and 200 µL of Wash Buffer was added into it. The column was centrifuged at 12,000 rpm for 30 seconds. Flow-through was discarded again. Then 200 µL of Desulfonation Buffer was added to the micro column in the same collection tube and incubated at room temperature for 20 min. Incubation was followed by centrifugation at 12,000 rpm for 30 seconds and resulting flow-through was subsequently discarded. 200 µL of Wash Buffer was added again to the Micro column in order to wash the converted product and centrifuged at 12000 rpm for 30 seconds. Once again the flow-through was discarded followed by another washing with 200 µL of Wash Buffer and centrifugation at 12,000 rpm for 60 seconds. The Micro column was then placed in a clean 1.5 mL micro-centrifuge tube and 10 µL of Elution Buffer was added into it and centrifugation was carried out at 12,000 rpm for 60 seconds. Elution was repeated once again. Eluted DNA was then stored at -20 ºC for downstream analysis.

3.5.5 Methylation Specific Primer Designing:

To find the sequence of promoter region of the gene, Ensembl Genome Browser (http://www.ensembl.org/index.html) was used to retrieve the sequence of human TNFα. To ensure that the selected region was the promoter sequence, the promoter sequence was copied and searched in UCSC BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start), "Human" was chosen as the desired genome and the promoter sequence was pasted. On the result page, clicked the first hit that opened the genome

browser Page. The query sequence was now aligned with UCSC genome sequence. Moreover, CpG island feature was turned on, and observation of CpG Island in the query sequences further confirmed it to be a promotor sequence.

Methylation Specific Primers were designed using an online designing tool known as MethPrimer (Li and Dahiya, 2002) accessed through http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi. Two sets of primers were used for analysis for methylation status. One pair for methylated DNA and other for unmethylated DNA. Table 3.2 shows the primer sequences for the two sets of primers and their respective product size.

3.5.6 Methylation Specific PCR Amplification:

The Bisulfite converted DNA was subjected to PCR amplification using Methylation Specific primers in order to analyze the methylation status of promoter region of TNFα. For each sample, two reaction tubes were prepared. One contained Methylated-Specific primer pair while the other contained un-methylation specific primer pair. Reaction mixture (20 µL) was prepared in 0.2 mL PCR tubes (Biologix, USA) by the addition of 2 µL of Bisulfite converted DNA, 2 µL each of Left and Right Methylated or Un-methylated-specific primers, 4 µL nuclease free water and 10 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, EU, Lithuania). The thermocycling conditions consisted of initial template denaturation at 95^{o} C for 10 minutes followed by 38 cycles of PCR amplification. Each amplification step further consisted of 3 steps: denaturation at 95^{o}C for 30 seconds, annealing at 57^{o}C for 1 minute and 1 minute at 72^{o}C for extension. Final extension was at 72^{o}C for 7 minutes. The PCR products were kept at 4^{o}C and analyzed on 2% agarose gel.

3.5.7 Gel Electrophoresis:

Gel Electrophoresis was carried out to analyze the methylation status of the TNFα promoter. A 2% agarose gel was prepared by dissolving 1.6 g of agarose in 80 mL of 1X TAE buffer and heated in microwave oven for 2 minutes. About 3 µL Ethidium bromide (0.5 µg/ml) was added to stain the DNA. The gel was allowed to solidify at room temperature in a gel casting tray. On solidification, the gel was transferred to buffer tank containing 1x TAE. About 7 µl of the amplified product was loaded on to the gel. The gel was the run at 100 volts for 35 minutes. Results were analyzed through Gel Documentation System (Dolphin Doc).

3.5.8 Statistical Analysis

For statistical analysis, version 5 of GraphPad Prism was used. Student's t-test and one-way ANOVA nonparametric Kruskal-Wallis test was used to test the hypothesis. P-values of, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ were considered to be significant.

Table 3.2: Primer sequences used in Methylation Specific PCR

Primer	Name	Sequence (5' – 3')	Product Size
MSP1	Forward Methylation specific primer	GAGATAGAAGGTGTAGGGTTTATTATC	130 bp
	Reverse Methylation specific primer	TACCTTTATATATCCCTAAAACGAA	
	Forward un-methylation specific primer	TAGAAGGTGTAGGGTTTATTATTGT	126 bp
	Reverse un-methylation specific primer	TACCTTTATATATCCCTAAAACAAA	
MSP2	Forward Methylation specific primer	TTAGAAGATTTTTTTCGGAATC	130 bp
	Reverse Methylation specific primer	TATCTCGATTTCTTCTCCATCG	
	Forward un-methylation specific primer	GGTTTAGAAGATTTTTTTTGGAATT	120 bp
	Reverse un-methylation specific primer	TCTATCTCAATTTCTTCTCCATCAC	

RESULTS

4.1 Collection and Analysis of Samples

Subcutaneous adipose tissue samples were collected from patients undergoing elective surgical processes at Holy Family Hospital, Rawalpindi and Bilal Hospital, Rawalpindi. Diagnosis of T2DM was made upon analysis of fasting plasma glucose levels and HbA1c. A total of 21 tissue samples were collected. Ten tissue samples were from people who were not diabetic and thus were used as a control group (Table 4.1). Seven tissue samples were from patients diagnosed with T2DM who were on oral anti-diabetic drugs (Table 4.2), while 4 were from T2DM who were on insulin therapy (Table 4.3).

4.2 Isolation of RNA from Adipose tissues and its Analysis

RNA was extracted from tissues following their homogenization in liquid nitrogen using the TRIzol® method. The quantity and quality of extracted RNA was determined by using Thermo Scientific NanoDrop 2000 Spectrophotometer and NanoDrop™ 2000 software at KRL Hospital, Islamabad. To determine the purity of RNA samples, $A_{260/280}$ was also measured.

4.3 cDNA synthesis

The quantified RNA was used to synthesize cDNA. The reverse transcription was carried out by using RevertAid Reverse Transcriptase (Thermo Scientific #EP0442). All of the RNA samples were used to synthesize cDNA at a concentration of 1000ng.

Confirmation of cDNA synthesis was done by PCR amplification of β-globin gene which is used as a housekeeping gene and is constitutively expressed in all cells.

Table 4.1 Adipose Tissue Samples from Non-Diabetic Control

S. No.	Age	Gender
1.	36	Female
2.	40	Female
3.	35	Male
4.	45	Female
5.	80	Male
6.	30	Female
7.	40	Male
8.	55	Female
9.	27	Female
10.	39	Male

Table 4.2 Adipose Tissue Samples from T2DM Patients without Insulin Treatment

S. No.	Age	Gender
1.	38	Male
2.	47	Male
3.	38	Female
4.	68	Male
5.	47	Female
6.	49	Female
7.	60	Female

Table 4.3 Adipose Tissue Samples from T2DM Patients on Insulin Therapy

S. No.	Age	Gender
1.	45	Male
2.	46	Male
3.	65	Female
4.	43	Female

4.4 Expression Analysis of TNFα

In order to confirm the expression of target gene in the samples, cDNA was used to optimize the amplification conditions of the target and control gene in conventional and real-time PCR. All the amplifications were run in duplicates to ensure the reproducibility of results.

cDNA template was used for target and control gene amplification. First a gradient PCR was used in order to determine the optimum annealing temperature for each of the primers. Results were confirmed by running the PCR products on 2% agarose gel at 100V for 30 minutes. A 50bp DNA ladder was used to confirm that the bands obtained were corresponding to the desired product and there was no non-specific amplification.

In order to optimize the amplification conditions on real-time PCR, 25ng of cDNA was used. Amplification plots for the target gene (TNFα) and control gene (β-actin) (Figure 4.1 and Figure 4.2) were optimized on Applied Biosystems 7300 Real Time PCR. Gene expression data was normalized to the expression levels of control or "housekeeping" gene. β-actin was used as a house keeping gene, a constitutive gene, expressing in all cells of the body. Any nonspecific amplification was also checked by examining amplification plots and dissociation curves, which confirmed that no secondary amplification took place at the optimized conditions.

4.5 Expression Analysis of TNFα relative to β-actin in T2DM and control samples

In order to find any differences in the expression levels of TNFα relative to β-actin between the three study groups, real-time PCR was used. Sybr Green chemistry was used in order to analyze the difference in expression through difference in fluorescence. Amount of

fluorescence correlates with the level of expression of the target gene in the sample. All the three groups i.e. T2DM patients without insulin treatment, T2DM patients on insulin treatment and non-diabetic controls were used to quantify difference in the expression of TNFα. β-actin was used as a housekeeping gene to normalize the expression of the target gene.

Expression of TNFα relative to the endogenous control gene, was found to be increased in T2DM patients without insulin and even higher in those on insulin therapy as compared to the control group (Figure 4.3). Non parametric one way ANOVA revealed that the difference in TNFα expression was statistically significant with a *P* value of 0.0250 (Table 4.4).

Moreover, the fold change in TNFα expression of the two experimental groups was found to be 2.43 fold higher in T2DM without insulin group and 3.66 folds higher in T2DM with insulin treatment group relative to is expression in the control group (Figure 4.4). However, this difference in fold-change was found to be statistically insignificant (*P* value > 0.05) (Table 4.5).

For the statistical analysis, GraphPad prism version 5 was used. All the values were represented as mean $\pm$SEM, P<0.05 for experimental vs control by using unpaired Student's t-test and one-way ANOVA analysis.

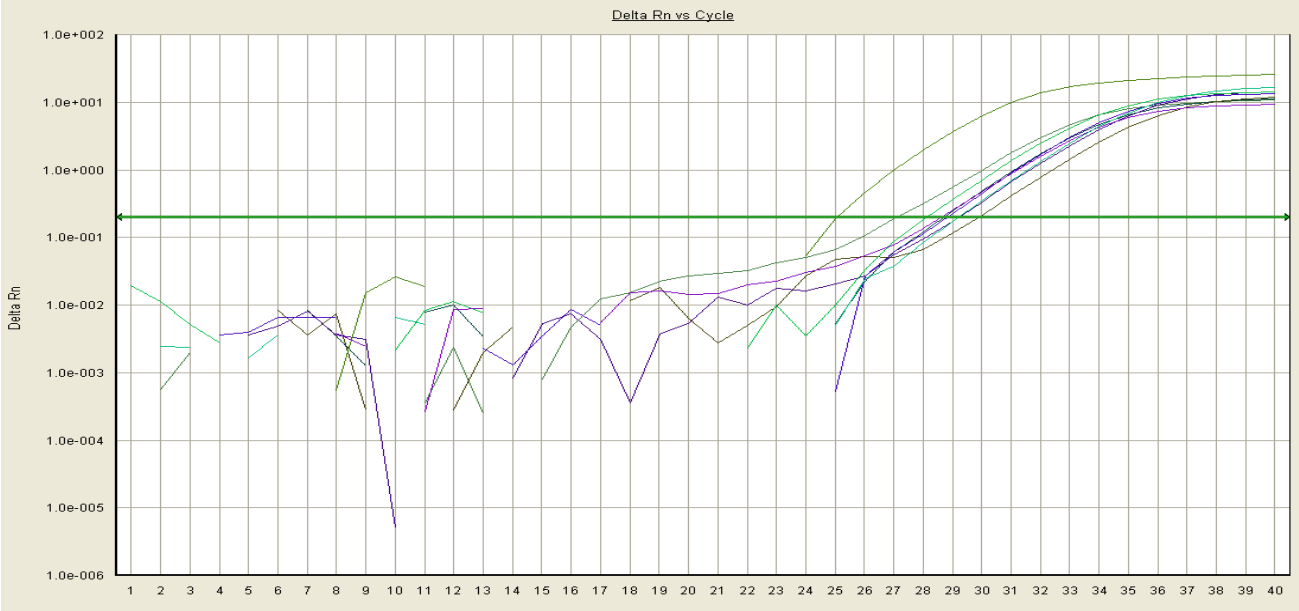

Figure 4.1 qPCR data analysis for TNF alpha: Amplification plot of TNFα plotted against cycle number on x-axis and delta Run on y-axis.

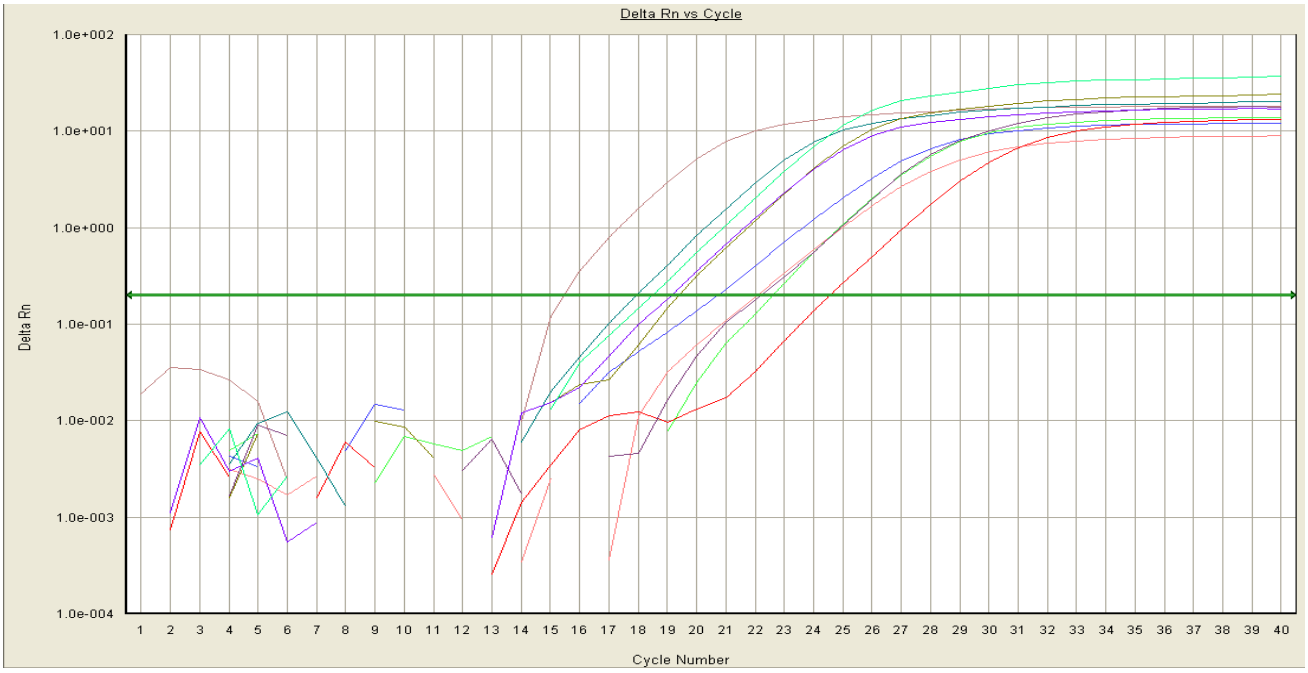

Figure 4.2 qPCR data analysis for β-actin: Amplification plot of β-actin plotted against cycle number on x-axis and delta Run on y-axis.

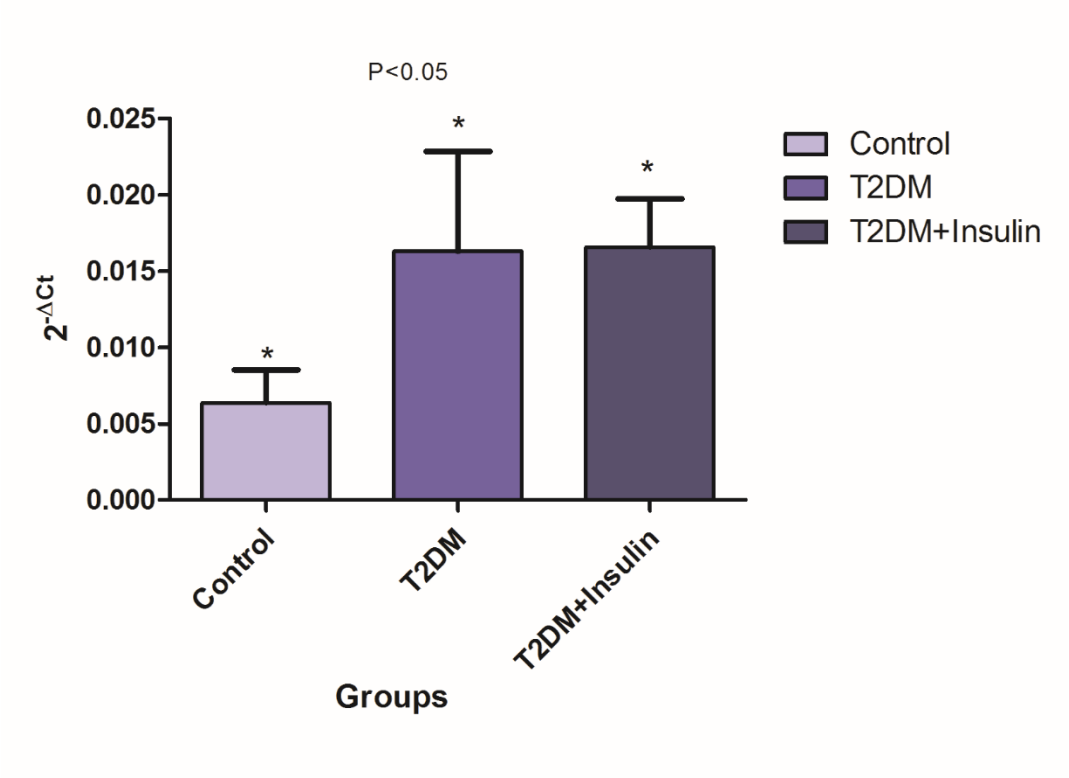

Figure 4.3 Expression analysis of TNF in experimental vs control group. The data is shown as the mean ± SEM,*P<0.05; **P<0.01, ***P<0.001. To confirm the constancy all samples were run in duplicates.

Table 4.4. One-way ANOVA (non-parametric) for expression of TNFα relative to β-actin ($2^{-\Delta Ct}$)

	Kruskal-Wallis Test		
P value	0.0250		
Exact or approximate P value	Gaussian Approximation		
P value summary	*		
Do the medians vary significantly (P <0.05)	Yes		
Number of groups	3		
Kruskal-Wallis statistic	7.378		
Dunn's Multiple Comparison Test (Post hoc)			
	Difference in rank sum	Significant P < 0.05	Summary
Control vs T2DMM	-5.222	No	Ns
Control vs T2DMM+Insulin	-8.622	Yes	*
T2DMM vs T2DMM+Insulin	-3.400	No	Ns

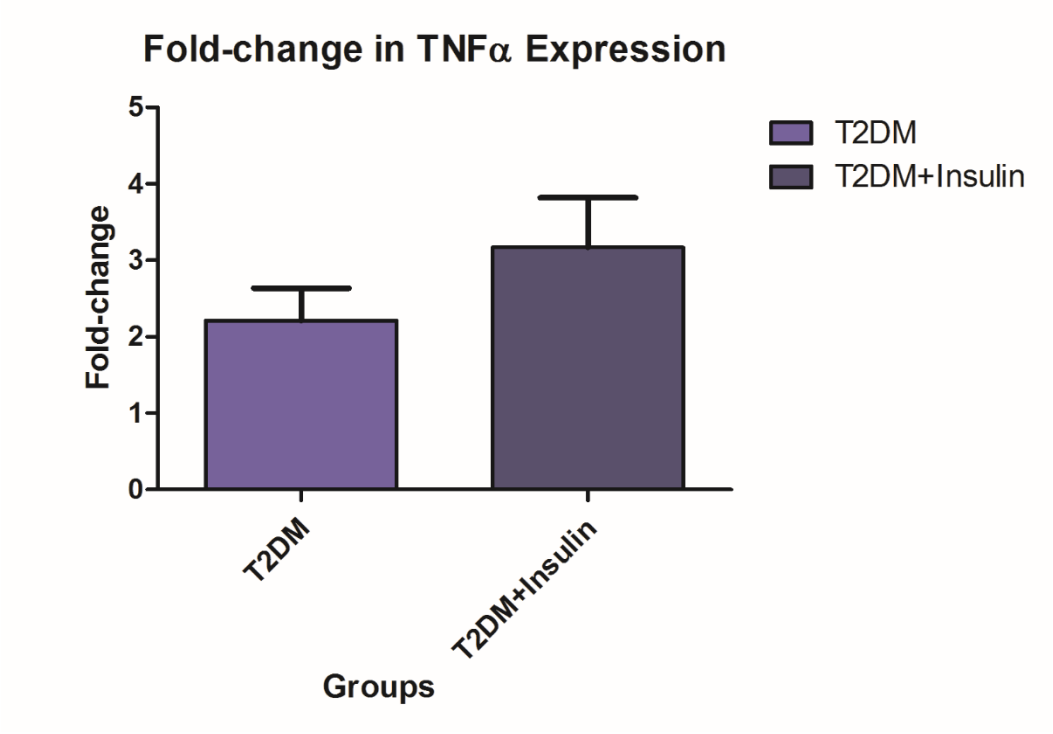

Figure 4.4 Analysis of fold-change in TNF expression between experimental and control groups. The data is shown as the mean ± SEM,*P<0.05; **P<0.01, ***P<0.001. To confirm the constancy all samples were run in duplicates.

Table 4.5. Unpaired Student's t-test for Fold-change in expression of TNF alpha relative to controls

Mann Whitney Test	
P value	0.3095
Exact or approximate P value	Exact
P value summary	Ns
Are medians signif different? (P < 0.05)	No
One- or two- tailed P value?	Two-tailed
Sum or ranks in column A, B	22 , 33
Mann-Whitney U	7.000

4.6 DNA Methylation Analysis

In order to find if changes in DNA methylation correlate with the changes in expression of TNFα between control and experimental groups, DNA methylation analysis was carried out upon the three groups of study. Methylation was analyzed at two loci in the promoter region of TNFα gene, hence two sets of primers were used, methylation specific primer 1 (MSP1) and methylation specific primer 2 (MSP2). Both of these primer pairs also had their corresponding unmethylation specific primers (U1 and U2, respectively) which were for amplification of the product if it was not methylated.

Results for MSP1 amplified promoter region of TNFα are shown in the gel images (Figure 4.5 and 4.6) and summarized in the table below (Table 4.6). These results indicate the methylation status of the TNFα promoter loci amplified by MSp1. The control group showed 50% methylation as compared to only 10% in T2DM without insulin and zero % in T2DM with insulin. Moreover, both experimental groups indicate an increase in partial methylation in comparison to the control group. A chi-square test was run on the data to test the hypothesis giving a p value <0.0001, thus suggesting that the difference in the methylation status of MSP1 specific loci between control and experimental groups was significant (Table 4.7). The percentage data is represented in the graph (Figure 4.9) thus showing a significant change in methylation status depicting association of unmethylation of one of TNFα loci with T2DM.

Results for MSP2 amplified promoter region of TNFα are depicted in Figure 4.7 and 4.8 and summarized in Table 4.8. These results indicate the methylation status of the TNFα promoter loci amplified by MSp2. Both experimental groups of patients of T2DM either on insulin or on oral-diabetics have their MSP2 specific loci partially methylated (100%)

as compared to the control group being only 60% partially methylated with 20% methylation. A chi-square test was run on the data to test the hypothesis giving a p value <0.0001, thus suggesting that the difference in the methylation status of MSP2 specific loci between control and experimental groups was significant (Table 4.9). The percentage data is represented in the graph (Figure 5.0) thus showing a significant change in methylation status depicting association of unmethylation of one of TNFα loci in T2DM.

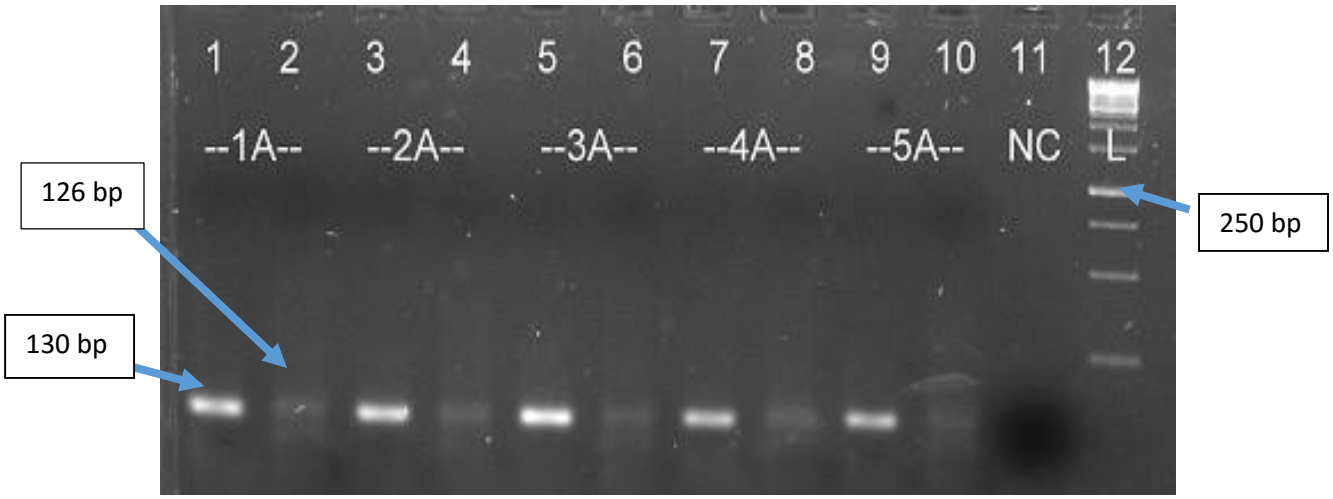

Figure 4.5 Methylation Analysis of MSP1 and U1 (A). The image shows PCR products for both experimental group A run on a 2% agarose gel. Lanes 1, 3, 5, 7, 9 show PCR products for methylation specific primer, while lanes 2, 4, 6, 8, 10 show PCR products for un-methylation specific primer. All the samples except for 7A, in this gel are partially methylated owing to the presence of bands in both wells corresponding to methylation and unmethylation specific primers. Sample 7A is methylated as there is no band in the well corresponding to un-methylation. ('A' represents T2DM patients without insulin therapy and NC is negative control)

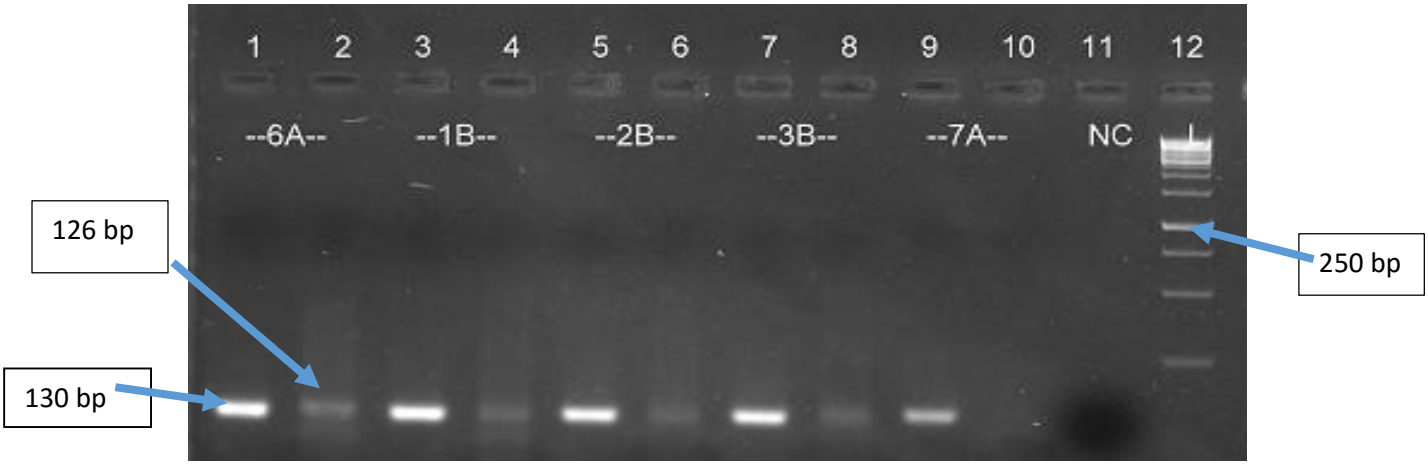

Figure 4.6 Methylation Analysis of MSP1 and U1 (B). The image shows PCR products for both experimental group A run on a 2% agarose gel. Lanes 1, 3, 5, 7, 9 show PCR products for methylation specific primer, while lanes 2, 4, 6, 8, 10 show PCR products for un-methylation specific primer. All the samples except for 7A, in this gel are partially methylated owing to the presence of bands in both wells corresponding to methylation and unmethylation specific primers. Sample 7A is methylated as there is no band in the well corresponding to un-methylation. ('A' represents T2DM patients without insulin therapy, 'B' represents T2DM patients with insulin therapy and NC is negative control)

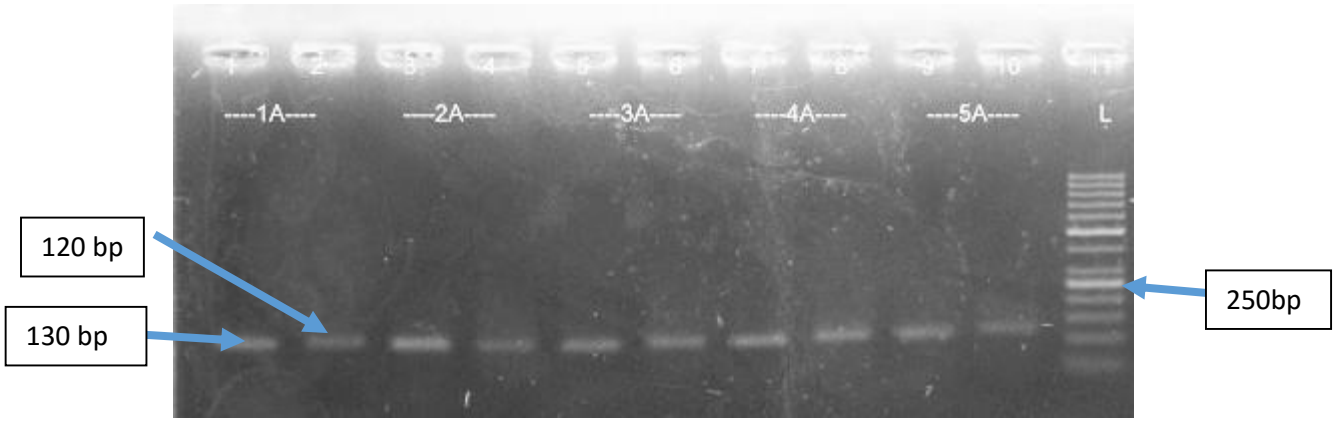

Figure 4.7 Methylation Analysis of MSP2 and U2 (A). The image shows PCR products for experimental samples group A run on a 2% agarose gel. Lanes 1, 3, 5, 7, 9 show PCR products for methylation specific primer, while lanes 2, 4, 6, 8, 10 show PCR products for un-methylation specific primer. All the samples in this gel are partially methylated owing to the presence of bands in both wells corresponding to methylation and unmethylation specific primers. ('A' represents T2DM patients without insulin therapy. NC is negative control)

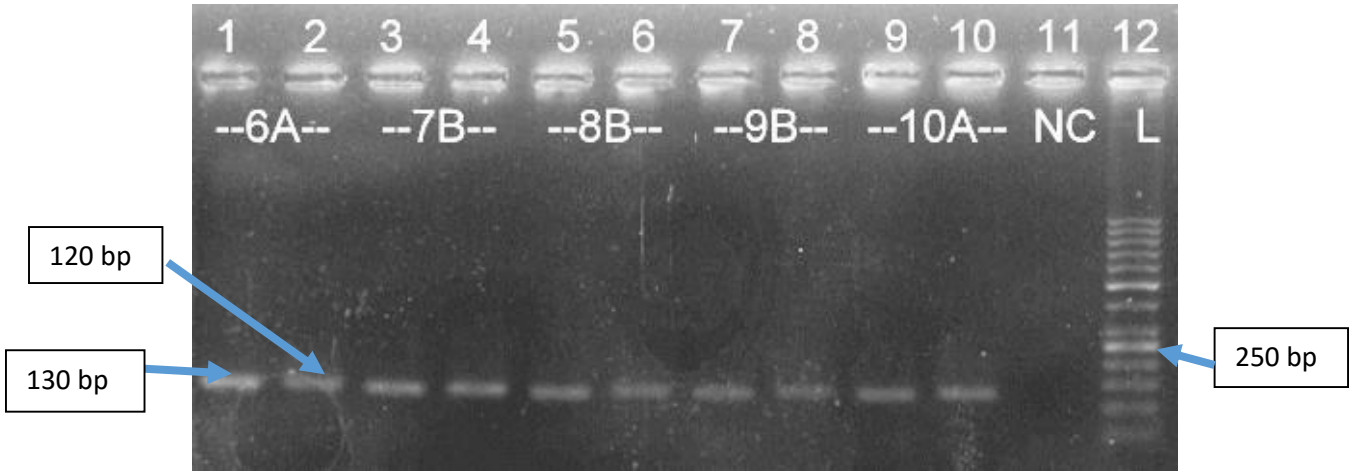

Figure 4.8 Methylation Analysis of MSP2 and U2 (B). The image shows PCR products for experimental samples group B run on a 2% agarose gel. Lanes 1, 3, 5, 7, 9 show PCR products for methylation specific primer, while lanes 2, 4, 6, 8, 10 show PCR products for un-methylation specific primer. All the samples in this gel are partially methylated owing to the presence of bands in both wells corresponding to methylation and unmethylation specific primers. ('A' represents T2DM patients without insulin therapy. NC is negative control)

Table 4.6 Methylation Status for TNFα promoter by MSP1

Groups	Methylation Status			p value
	Methylated	Partially Methylated	Un-methylated	
Type 2 Diabetics without Insulin Treatment	1 (10%)	6 (90%)	0%	<0.0001
Type 2 Diabetics on Insulin Treatment	0%	4 (100%)	0%	
Controls	5 (50%)	5 (50%)	0%	

Table. 4.7 Chi-square contingency test for MSP1 specific PCR

Chi-square, df	79.29, 2
P value	< 0.0001
P value summary	***
One- or two-sided	NA
Statistically significant? (alpha<0.05)	Yes

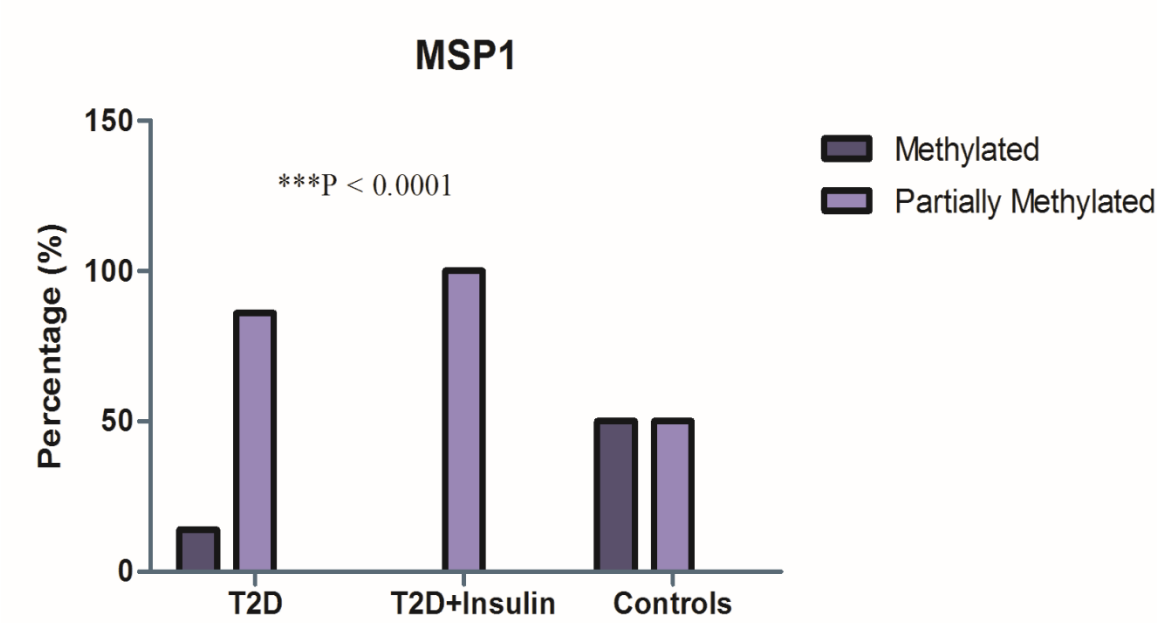

Figure 4.9 Methylation Status for MSP1 specific PCR. The data is presented as percentage of control and experimental samples. ***P<0.0001.

Table 4.8 Methylation Status for TNFα promoter by MSP2

Groups	Methylation Status			p value
	Methylated	Partially Methylated	Un-methylated	
Type 2 Diabetics without Insulin Treatment	0	7 (100%)	0	<0.0001
Type 2 Diabetics on Insulin Treatment	0	4 (100%)	0	
Controls	2 (20%)	6 (60%)	2 (20%)	

Table 4.9 Chi-square contingency test for MSP2 specific PCR

Chi-square, df	92.31, 4
P value	< 0.0001
P value summary	***
One- or two-sided	NA
Statistically significant? (alpha<0.05)	Yes

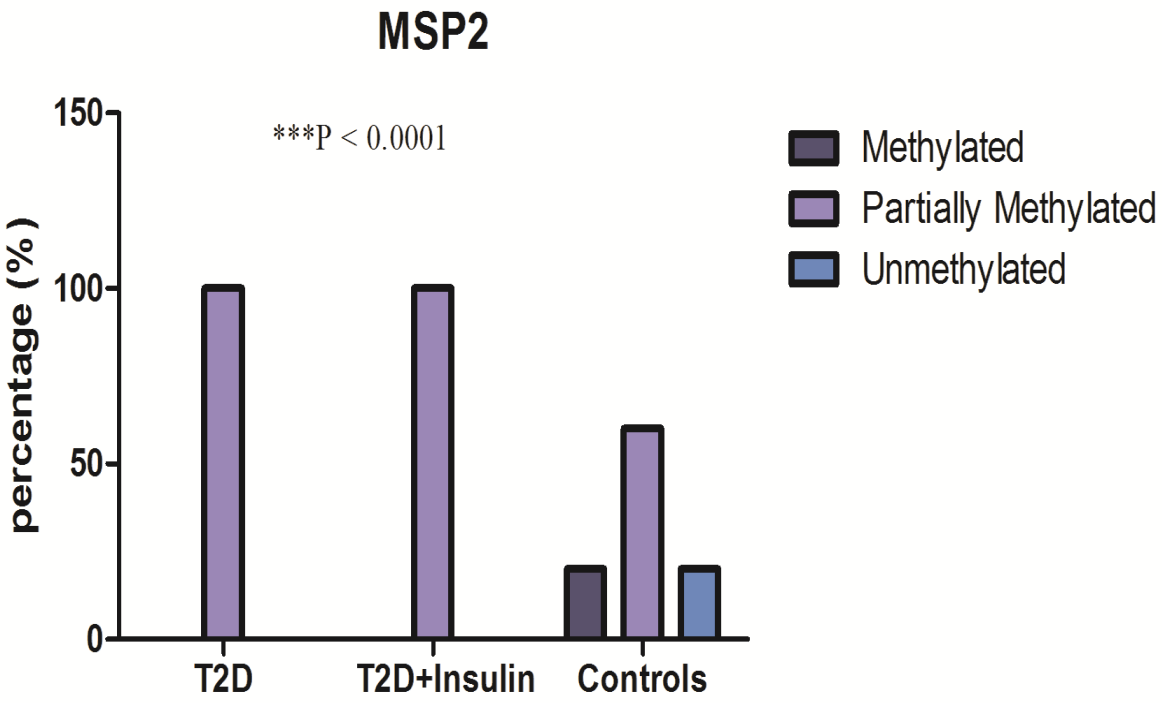

Figure 5.0 Methylation Status for MSP2 specific PCR. The data is presented as percentage of control and experimental samples. P<0.0001.

DISCUSSION

Diabetes refers to a class of disease characterized by hyperglycemia due to insufficient insulin production by the pancreas, inability of the target cells to respond to insulin action or both (Groop and Lyssenko, 2009). The chronic hyperglycemia makes the individual highly susceptible to developing damage, dysfunction and failure of organs like eyes, kidneys, nerves, blood vessels and heart. According to estimates of worldwide prevalence figures, there were 382 million individuals affected with diabetes in 2013 and this number is predicted to rise to 592 million by 2035. Type 2 Diabetes Mellitus is the most prevalent form of diabetes, constituting almost 80%-90% of the cases. It is characterized by hyperglycemia due to inability of the pancreatic beta-cells to increase insulin synthesis and secretion to compensate for insulin resistance in the target tissues. T2DM has been known to have a familial disposition. Individuals having one affected parent are at a 40% risk of developing T2DM while those with both parents affected have a 70% risk (Kobberling and Tillil, 1982).

Adipose tissue has long been recognized not only as a site of excess fat storage but also as a site of secretion of various hormones and cytokines, adipokines. Therefore, adipose tissue acts as a regulator of metabolic processes. High caloric diet and buildup of excess fat depots leads to abnormalities in adipocytes function in secretion of adipokines and storage of fats. Adiposity starts an inflammatory response in adipocytes as a result of which they secrete inflammatory cytokines including TNFα and MCP-1 (Lagathu *et al.*, 2006). As a result of inflammatory response macrophages infiltrate the adipose tissue (Curat *et al.*, 2004; Xu *et al.*, 2003b) resulting in an even further increase in secretion of inflammatory cytokines including TNFα which subsequently increases the rate of lipolysis and thus results in high

levels of circulatory NEFAs which enter the target cells e.g. muscles and make then insulin resistant, hence, affects adipocyte metabolism crucially, dulls down insulin signaling in adipocytes and target tissues and also affects the process of adipogenesis (Guilherme *et al.*, 2008). Studies have shown that in obese and insulin resistant states, TNFα inhibits phosphorylation of IR after insulin binds to the receptor, thereby inhibiting subsequent phosphorylation of IRS-1 and downstream signaling in response to insulin (Hotamisligil *et al.*, 1994; Liu *et al.*, 1998).

Increased expression of TNFα mRNA has been reported in adipose tissue of obese and insulin resistant humans (Hotamisligil *et al.*, 1995); however, this finding has not been consistent (Kern *et al.*, 2001). Moreover, higher amounts of TNFα protein have been demonstrated to be present in skeletal muscle of insulin resistant individuals (Saghizadeh *et al.*, 1996). This suggests that it might not be adipocytes altogether but surrounding stromal and macrophages that synthesize and secrete most of the TNFα (Weisberg *et al.*, 2003).

T2DM is a progressive disease marked by insulin resistance as well as insufficient secretion of insulin owing to βcell failure overtime. Studies have demonstrated that by the time diagnosis occurs, βcell function has already reduced by 50%-60% (Kahn and Halban, 1997; Ward *et al.*, 1984). Individuals genetically predisposed to T2DMM are more susceptible to βcell dysfunction leading to glucolipotoxicity (Popa and Mota, 2013). β-cell dysfunction occurs owing to chronic hyperglycemia and dyslipidemia which occurs as a result of increased cytokine production and dysfunction of adipocytes (Moller, 2000).

This study was conducted to find differences in expression of TNFα between control, T2DM without insulin and T2DM with insulin treatment in order to find out if βcell failure

is characterized by any change in TNFα levels. Findings of this study have demonstrated that expression of TNFα shows a fold-change of 2.43 and 3.66 in T2DM individuals without and with insulin treatment respectively relative to individuals not suffering from T2DM. However, the fold-change was not found to be statistically significant. This suggests that β-cell failure requiring insulin therapy, may not be characterized by high levels of TNFα.

Any diseased state, T2DM in the context of this particular study is associated with changes in gene expression allowing the deviation from a balanced and normal state of function. Epigenetics has emerged as a new framework for researching gene expression changes in environmental-related diseased states like T2DM (Levy *et al.*, 1998). Epigenetics is defined as heritable and reversible changes in gene expression without any change in the primary DNA sequence. Histone modifications and DNA methylations of CpG (cytosines followed by guanine) are two major epigenetic mechanisms regulating gene expression. CpG islands are usually located in or near promoters for about 50% of human genes (Caiafa and Zampieri, 2005; Campion *et al.*, 2009). Methylation of these CpG sites renders them transcriptionally repressed (Esteller, 2008). The objective of this study was to investigate whether the basal methylation profiles of specific CpGs located in TNF-alpha promoters, as measured by methyl specific PCR in subcutaneous adipose tissue from T2DM individuals on and without insulin treatment could correlate with changes in expression of TNFα in these states when compared to non-diabetic individuals. Changes in secretion of TNFα by macrophages are mediated by epigenetic modifications that occur during the process of monocyte differentiation (Sullivan *et al.*, 2007). Furthermore methylation

differences in TNF-alpha promoter region have also been associated with distinct responses to a low-calorie diet in blood (Campion *et al.*, 2009).

The results of the research indicated that as compared to the control subjects, TNFα promoter was significantly less methylated at the two loci under study in T2DM subjects with or without insulin treatment. This correlates with increased expression of TNFα relative to β-actin in the three groups of study. Hence, showing that the change in TNFα expression is associated with changes in methylation pattern of its promoter region in type 2 diabetes. Nonetheless, the methylation status of the TNFα promoter can possibly be used as a predictor of development of T2DM and subsequent β-cell failure in comparison to healthy individuals.

CONCLUSION

This study has shown that the expression of TNFα in the two T2DM study groups relative to a housekeeping gene, β-actin is significantly higher than that of control group. However, the fold change in expression of TNFα between the two experimental groups differing by insulin treatment is not significant, thus indicating that TNFα may not be implicated in β-cell failure. The DNA methylation pattern of TNFα promoter by the technique of methylation specific PCR after Bisulfite conversion has shown significant reduction in methylation relative to the control study group. This change in methylation pattern is most certainly due to the disease-onset which modifies the epigenetic landscape resulting in enhanced expression of TNFα with regards to Type 2 Diabetes.

FUTURE PROSPECTS

After this initial approach on epigenetic biomarkers search, further studies are required to look for extensive epigenetic regulation of other important adipokines involved in metabolic regulation and T2DM pathogenesis. With regards to the role of TNFα, its DNA methylation patterns could also be studied in the macrophages infiltrating adipocytes in wake of an inflammatory response. These DNA methylation markers could be used in microarray screening and could also be used as predictor of T2DMM and progressive β-cell failure.

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