# Association Analysis of Genetic Polymorphism of *TCF7L2* Gene in Patients of Type 2 Diabetes Mellitus (T2DM)

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**H-12, ISLAMABAD, PAKISTAN**

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A thesis submitted in partial fulfillment of the requirements for the degree of MS Healthcare Biotechnology

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**2019**

# DECLARATION

I certify that this research work titled "Association Analysis of Genetic Polymorphism of *TCF7L2* Gene in Patients of Type 2 Diabetes Mellitus (T2DM)" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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______________________________

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**“Education is the kindling of flame, not the filling of the vessel”**

**Socrates**

**Dedicated to my incredible parents for their enormous support and relentless efforts for turning every stumbling block into stepping stone.**

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**Hadiqa Mubashir**

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## List of Acronym

| | | | |
|---|---|---|---|
| ASPCR | Allele specific polymerase | RPG | Random plasma glucose |
| T2DM | Type 2 Diabetes Mellitus | FGP | Fasting glucose plasma |
| NIDDM | Non insulin dependent chain reaction | ADA | American Diabetes Association |
| ROS | Reactive oxygen species | TCF | TCF family (Transcription factor family) |
| IGT | Impaired glucose tolerance | TSS | Transcriptional start sites |
| IR | Insulin Resistance | HMG | High mobility group |
| DPP-IV | Dipeptidyl-peptidase-IV | RPG | Random plasma glucose |
| IR | Insulin Resistance | FGP | Fasting glucose plasma |
| RYGB | Roux-en-Y gastric bypass | CBP | CREB-binding protein |

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# ABSTRACT

Rate of occurrence of non-insulin dependence diabetes mellitus (NIDDM) is burgeoning at an alarming rate every year. Type 2 diabetes mellitus (T2DM) is a condition in which body doesn't respond efficiently to insulin or develops resistance against insulin. It is ascribed to both genetic and environmental factors. The interplay of genetic and environmental factors make it difficult to pinpoint the exact mechanism and to implicate the pancreatic beta cells for progression of diabetes. However, there is a growing interest in involvement of genetics in pathogenesis of T2DM. This study was conducted to ascertain association analysis of SNPs (*rs 12255372 and rs 4506565*) of *TCF7L2* gene with diabetes in a total of 186 subjects. For this, allele specific polymerase chain reaction was carried out to amplify SNPs of *TCF7L2* and to examine their prevalence in population. A significant association was seen between these SNPs and T2DM. Predisposition to diabetes increase with presence of GT genotype in *rs 12255372* with OR= 2.25, 95% CI= 1.2-4.07 , P=0.003** and AT genotype in *rs 4506565* with OR= 1.35, 95% CI=0.7-2.4 , P=0.002**.The allelic distribution shows the high prevalence of risk allele T in experimental than in control. Moreover, computational analysis was performed to analyze the underlying mechanism of *TCF7L2* SNPs and their role in increasing the susceptibility of T2DM. No significant change was seen in splicing mechanism when observed through softwares such as Splice View, Alternate Splice Site Predictor (ASSP), Exon Splice Enhancer (ESE). However, alteration of two motifs (Gfi1, Gfi1b) are seen in case of *rs4506565* and alteration of five motifs in case of *rs12255372* when observed through HaploReg software. Another software, Ensembl Variant Effect Predictor (VEP) has predicted *rs12255372* as a risk allele and thus shows its clinical significance in pathogenesis of T2DM.

# *CHAPTER#1 INTRODUCTION*

# INTRODUCTION:

## 1.1. TYPE 2 DIABETES MELLITUS (T2DM):

Type 2 Diabetes mellitus (T2DM) is a condition of high glucose level that occur because of defective insulin secretion or developed insulin resistance in body. The burgeoning rate of occurrence, morbidity and mortality of T2DM is growing at a very alarming scale worldwide (Phillips, 2016).

The most prevalent form of diabetes is T2DM and pathogenesis of T2DM involve genetic, non genetic and environmental factors (Kharroubi & Darwish, 2015). Multifactorial nature of T2DM encompasses multiple genetic factors which affect insulin production, insulin resistance, glucose hemostasis, and other environment influencers such as obesity, aging, binge eating which ultimately leads to hyperglycemia and subsequently cause macrovascular and microvascular complications (Tuomilehto et al., 2001). Microvascular complications entail impairment or dysfunction of small vessels or arteries of kidney, eyes, nerves and macrovascular complications include damage to large vessels such as arteries of heart, brain, limbs and sensory loss (Chawla, Chawla, & Jaggi, 2016).

Type 2 diabetes commonly known as non-insulin dependent diabetes mellitus (NIDDM) is also considered to be a type of idiopathic diabetes because of it's inability to inhibit diabetic ketoacidosis (DKA) (Erondu, Desai, Ways, & Meininger, 2015). Also, the insulin resistance feature of T2DM could be because of the misfunctioning of immune cells (that instead of acting as a shield against foreign invaders, attack their own body cells), linking the possibility of T2DM to be an autoimmune disorder rather than just a metabolic disorder but so far no strong link has been established and identified between NIDDM and autoimmunity because of the heterogeneous and polygenic nature of T2DM (Itariu & Stulnig, 2014).

### 1.1.1. Pathophysiology and pathogenesis of T2DM:

Malfunctioning of lipids and carbohydrates metabolism lead to development of metabolic disorders such as T2DM. Organs such as kidney, adipose tissues, liver, skeletal tissues play a pivotal role in maintaining blood glucose level in blood. Process of glucose formation and glucose breakdown is controlled by organs and multiple signaling pathways are involved in this. (Grytsay, 2017). These pathways are indispensable for nutrient metabolism. Process of gluconeogenesis mainly occur in liver and to a lesser extend in kidney and causes glucose formation. Excessive production of glucose from non carbohydrate sources contributes in hyperglycemic condition (Y. Wang et al., 2012).

Pathogenesis of T2DM involves both genetic and acquired factors. Pancreas synthesize insulin hormone that takes up glucose from body and maintains blood glucose level or glucose homeostasis (Ozougwu, Obimba, Belonwu, & Unakalamba, 2013). Beta cells of pancreas produce insulin hormone which needs facilitation to move inside cell membrane. Glucose transport mechanism especially glucose transporter 2 (GLUT2) facilitates movement of glucose inside and outside of cell. GLUT2 is basically a transmembrane carrier protein (Gautier, Choukem, & Girard, 2008).

Moreover, endocrine cells of intestine release incretin hormones. They are released as a result of ingestion of food. There are two types of these incretin hormones; glucagon like peptide 1 (GLP1) and GIP (glucose-dependent insulinotropic peptides). (Nolan & Færch, 2012). K cells of small intestine synthesize GIP1 while the L cells usually found in large intestine produce GLP1 hormone. These incretin hormones are important for insulin production, glucagon inhibition and conversion of incretin into inactive metabolites by action of enzymes DPPIV (dipeptidyl-peptidase-IV) (Hajiaghaalipour, Khalilpourfarshbafi, & Arya, 2015).

One of the principle contributor of DM is dysfunction of beta cells and insulin insensitivity. Proper interaction between insulin sensitive cells and beta cells is essential for amino acids, fatty acids, glucose uptake. There are myriad reasons of malfunctioning of beta cells. It could be mutations in genes of insulin regulation, synthesis or Islet Amyloid Polypeptide (IAPP) or amylin (Hjuler, Gydesen, Andreassen, Karsdal, & Henriksen, 2017). Amylin is secreted simultaneously along with insulin. It is a residue of 37 amino acids and plays a role in insulin regulation. Whenever body is unable to utilize insulin it leads to an elevated level of insulin and amylin since they are co synthesized. So regulation is affected and leads to progression of diabetes (Baram, Atsmon-Raz, Ma, Nussinov, & Miller, 2016). However, insulin sensitivity occur when insulin is disturbed and cause lowering of glucose level in adipose tissues and an enhanced production of glucose in liver. Adipose tissues are the prime site for lipolysis and causes release of non-esterified fatty acids (NEFA) so basically provide stored energy in time of need. However, if by any means insulin is unable to inhibit lipolysis process in adipose cells, the repercussion would be an elevated fatty acids which in turn would damage beta cell function and cause hindrance in insulin production and would trigger gluconeogenesis (Kusminski, Bickel, & Scherer, 2016).

Defect in pancreas that produce insulin hormone affects responsiveness to glucose and cause a decrease in glucose uptake and ultimately hyperglycemia in body. The condition of DM involves a defective insulin production and insulin resistance. The first phase of impaired insulin synthesis leads to Impaired glucose tolerance (IGT) which is basically a prediabetic condition. In this, if condition continues and no precautionary measures are taken it leads to glucose toxicity and ultimately to insulin resistance and loss of control of blood glucose level. A completely degenerative pancreatic cell will cause loss of beta cells and an increased blood glucose level (Tiwari & Rao, 2002).

The second phase which is insulin resistance has links with molecular genetics and lifestyle. The manifestation of insulin resistance usually increase many folds before progression of diabetes. Polymorphism in around 30 genes which include genes of insulin, Insulin receptor substrate (IRS), adrenergic receptor genes (ARG), transcription factor 7 like 7 (*TCF7L2*), uncoupling protein genes (UCP) and many other have found to be associated with insulin resistance. Also, adipokines or adipocytokines which are released by adipose tissues and their varying circulating concentration causes insulin resistance. Likewise, leptin, tumor necrosis factor (TNF), resistin play major role in regulating insulin signaling pathway and imbalance can cause insulin resistance (Hajavi et al., 2017). High intake of fat containing food causes an increase level of fatty acids and contributes in development of insulin resistance (Kohei, 2010).

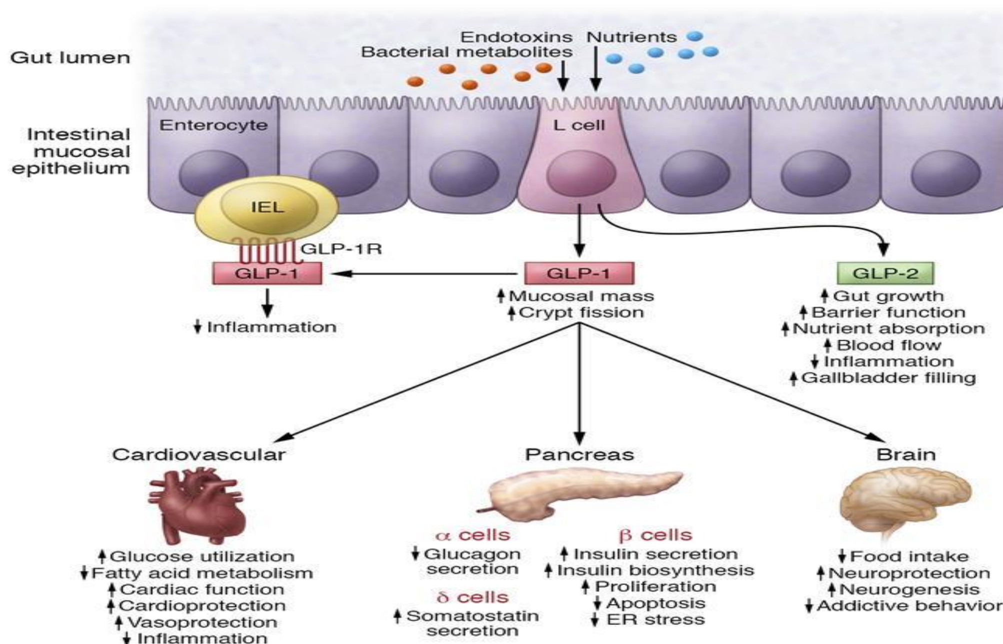


***Figure 1.1***. *The synthesis of GLP-1 from intestine in response to endotoxins, nutrients (Drucker, Habener, & Holst, 2017)*

**Linkage of oxidative stress with diabetes:**

Free radicals are generated in condition of oxidative stress. Oxidative stress occur when there is mis proportion in numbers of free radicals generated by body and body's ability to get rid off these free radicals. Oxidative stress plays a prime role in progression of diabetes type 2 because of the excessive production of super oxides by mitochondria. The reactive oxygen species (ROS) could be deleterious for proteins, lipids (Asmat, Abad, & Ismail, 2016). The underlying reason of over production of free radicals is high intake of fatty and sugary food and an inactive way of living. Like ROS, there are RNS (reactive nitrogen species) which are involved in signaling transduction pathway (Cai & Kang, 2001). They can play dual role; ROS/RNS are generated in insulin production pathways and impose a positive impact while the same ROS/RNS are also involved in negative regulation and cause insulin resistance (Henriksen, Diamond-Stanic, & Marchionne, 2011).

### 1.1.2. Epidemiology of type 2 diabetes:

Diabetes mellitus is a fastest growing global health issue. DM termed as non-transmissible or non-communicable disease (NCD) is among one of the leading causes of death and its prevalence is exponentially increasing throughout the world. (P. Z. Zimmet & Alberti, 2016). Precisely, it is ninth major cause of death globally. (Zheng, Ley, & Hu, 2018). According to international diabetes federation (IDF) report 371 million people are suffering from diabetes and countries with highest prevalence of diabetes are United States, China, India.(P. Zimmet, Alberti, Magliano, & Bennett, 2016). According to Centre for disease control and prevention (CDC) report, around 100 million United states residents are diagnosed with diabetes. (Kharroubi & Darwish, 2015). There are multiple drivers of diabetes along with genetics which include binge eating, sedentary lifestyle, lack of exercise, alcohol consumption, uncontrolled diet, high consumption of sugary or high caloric diet, epigenetics, aging, stress etc. all these

factors increase susceptibility to diabetes. (Kharroubi & Darwish, 2015). One of these factors overweight, is strongly linked to diabetes. Statistics mark that around 80 percent diabetic patients are obese (Hawley & McGarvey, 2015).

According to studies, impact of diabetes on Asia and western Pacific is huge and predominantly its prevalence in Asia is around 60 percent and most common in China, India, Pakistan, Bangladesh and Nepal (Nanditha et al., 2016). As claimed by population based statistics, in Pakistan the overall prevalence of type 2 diabetes mellitus (T2DM) is from 9 percent to 11 percent and is expected to raise as high as 16 percent if left unnoticed (Aftab et al., 2017). According to the current statistics, Pakistan stands at number 7th among countries most affected by T2DM. (Basit, Fawwad, Qureshi, & Shera, 2018). Gender based distribution in males and females in Pakistan according to national diabetes survey is 11.3 percent and 9.2 percent (Shahzad et al., 2018).

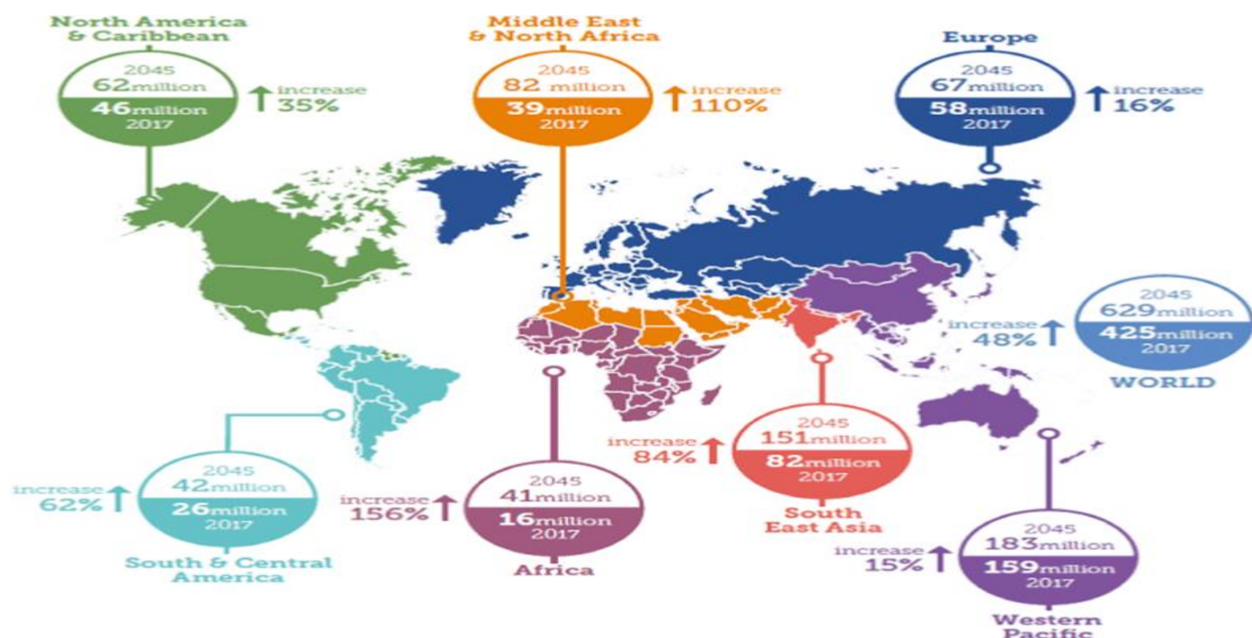


***Figure 1.1.2:*** *Worldwide distribution of Diabetes and prediction of percentage increase by 2045. (Glovaci, Fan, & Wong, 2019).*

## 1.2. T2DM AND CORELATED RISK FACTORS:

### 1.2.1. GENETIC DETERMINANTS:

Involvement of genes lead to an exponential increase in predisposition to T2DM. (Fuchsberger et al., 2016). Family history and ancestral pedigree play a prime role in susceptibility of DM. The probability of developing T2DM elevate to 45 percent if inherited from single parent (Tillil & Köbberling, 1987). According to lineage studies, the risk associated with developing T2DM in identical twins is more than 75 percent however in non identical twins the ratio is very less and stands at 30 percent only (Medici, Hawa, Ianari, Pyke, & Leslie, 1999). Below is the listicle of susceptible genes and their associated risk factor with T2DM. They include;

1. ***SLC2A2*** that is solute carrier family 2 member 2. It facilitates in transportation of glucose across the body. It plays key role in absorption of glucose in kidney. This gene basically encodes transmembrane carrier proteins that aids glucose transportation. Any mutation in this gene causes deregulation of insulin transportation. (Sansbury et al., 2012)
2. ***PPAR Gamma*** encodes proteins because their function is similar to that of a transcription factor. These peroxisomes proliferator genes are involved in adipogenesis. Thus establishing the link between obesity and T2DM. There is a downregulatory effect of PPAR in case of DM (Lecarpentier, Claes, Vallée, & Hébert, 2017).
3. ***KCNJ11*** is a voltage gated potassium channel which is under the command of G proteins. G proteins play a crucial role in controlling the influx of molecules and act as switches. KCNJ11 gene act as a guide for making of KATP subunits which are located in beta cells of pancreas and maintain the flow of glucose as demand of body. Mutation in this would affect a major subunit of potassium channel that is Kir6.2 and result in hyperglycemia (Breidbart et al., 2018).
4. ***NOTCH*** genes have a paramount importance in notch signaling pathways which are mediators in various metabolic pathways. There are notch ligand and receptors which play

a prime role in cellular communication, proliferation and ultimately in deciding destination of cells. Improper activation of notch pathways lead to elevated levels of glucose and deregulation of processes such as gluconeogenesis and lipogenesis (Sukmawati et al., 2016).

5. ***TCF7L2*** gene encode transcription factor proteins. It stands for transcription factor 7 like 2. It plays a crucial role in beta catenin or Wnt signaling pathway. These pathways are imperative in embryonic development, transcription of genes and cell to cell adhesion. Overexpression of *TCF7L2* affect metabolism of glucose. However, expression of GLP1 is affected by amount of *TCF7L2* protein (Shu et al., 2015).
6. ***IRS*** which are insulin receptor substrate are involved in pathogenesis of T2DM. These genes encode proteins that act as scaffold proteins and transfer signals from outside o inside cell hence involved in signal transduction. Any mutation in these protein could cause a decrease in insulin response (Lavin, White, & Brazil, 2016).
7. ***HNF1A*** is hepatic nuclear factor-1-alpha, this gene perform the function of a molecular switch by switching on or off expression of other genes. In childhood it permits pancreas to produce require amount of insulin however with onset of adulthood malfunctioning begins leading to an insufficient amount of insulin. (Pavić et al., 2018)
8. ***WFS1*** gene is considered to have paramount significant because it produces a protein that maintains the level of calcium in cells. This gene is found in many organs including kidney, liver, pancreas and perform important cellular functions. Since it is situated in endoplasm reticulum of cell so it facilitates the proper folding of protein. In pancreas, if proper folding is not maintained by this gene it would cause fragmentation of beta cells and subsequently lowering the insulin secretion.(Pedroso et al., 2015)

9. ***IGF2BP2*** gene make protein product of insulin like growth factor which belong to the family **of** IGF mRNA binding protein (IMP). This act by attaching to the insulin growth receptor and modulating translation of IGF. Any polymorphism would lead to alternative splicing affecting protein expression (Rao et al., 2016).
10. ***HHEX*** genes which are significant in several developmental pathways encode transcription factor proteins. These haematopoietically expressed homeobox affect beta cell differentiation and are part of Wnt signaling pathway. (Horikoshi et al., 2007)
11. ***FTO*** genes encode fat mass and obesity associated protein. The immediate effect of this protein product is an increase in body fat which inadvertently link to diabetes by altering glucose homeostasis. (Naaz, Kumar, & Choudhury, 2018)

### 1.2.2. Environmental determinants:

T2DM is widely influenced by multiple environmental and epigenetic factors. Some of them are as follow:

### I. Stress or hypertension:

In state of stress, body respond differently and releases hormones such as cortisol and adrenalin. These stress hormones elevate heart rate and cause a surge in body's glucose level by liver in order to deal with the stress situation and act as reservoir which provide fuel thus raising blood glucose level. Continuous stress cause hypertension and studies have established links between hypertension and diabetes because they have some pathways that are found in this and in both cases there is an increased amount of CRP (C REACTIVE PROTEIN) which is a signal of inflammation in body (Cornelis, Zaitlen, Hu, Kraft, & Price, 2015). Any change in end products of oxidative stress, RAAS (renin-angiotensin-aldosterone system) , SNS (Sympathetic nervous system),

Peroxisome proliferator-activated receptors (PPARs) would alter blood pressure and glucose level (Lago, Singh, & Nesto, 2007).

## II. Unhealthy/ processed food:

Any confectionery or processed product when taken in large amount have deleterious effects on health. These foods have harmful effect on body's natural absorption and digestion. These foods are bereft of natural nutrients and fibers. These foods cause random fluctuations in blood glucose level because of high fat and sugar content they are quickly absorbed in blood and leads to the generation of bad cholesterol (Baker & Friel, 2014).

## III. Physical inactivity:

Researchers have found a strong link between physical activity and development of T2DM. There is a precipitous increase in plasma glucose level after having a meal that is usually refer as PPG (postprandial glucose). It is a determinant of T2DM and when there are large spans of reduced physical activity and no exercise it leads to accumulation of plasma glucose and body's reaction to glucose increase manifolds. Glucose homeostasis is disturbed by lack of exercise and physical activity (Venables & Jeukendrup, 2009).

## IV. Obesity:

There is an alarming rise in obesity worldwide. According to some recent statistics around 87 percent patients of T2DM are overweight. And their weight reduction can cause a decline in developing T2DM of around 5-10 percent. (Tuomilehto et al., 2001). The accumulation of fatty acids in body makes it vulnerable for cytokines that are released by cells under some inflammation. These cytokines inhibit insulin signaling

pathways and making them unable and ultimately resistant. The unavailability of insulin causes a permanent spike in glucose level (Park, Sadanala, & Kim, 2015).

### V. Age:

The age bracket that is more vulnerable for type 2 diabetes is around or above middle age. Sedentary lifestyle and overweight are somewhat consistent features of T2DM so aging causes a decline in insulin response and decrease active functioning of beta cells. Another linkage is between mitochondrial decline and age. Mitochondria that is known as the powerhouse of cell manages several process including cell-cell communication, apoptosis etc. With age a gradual decline in mitochondria is seen that lead to inability of cells to manage with energy demands (Tan, Forbes, & Cooper, 2007).

### VI. PCO (polycystic ovary):

In PCOs, endocrine system causes an uninterpreted synthesis of androgen hormone that leads to malfunctioning of ovary. Both T2DM and PCO have some common risk determinants. Both are induced by obesity. Insulin resistance is also common in both disorders. Insulin resistance leads in contribution of development of PCOs (Conn, Jacobs, & Conway, 2000).

## 1.2.3. LIFESTYLE IMPACT:

The predisposition to diabetes increases exponentially due to one's adapted lifestyle. Lack of physical activity, sedentary lifestyle, smoking, uncontrolled alcohol consumption, utilization of saturated fatty acids (Kriska et al., 2018).

Some chemicals or toxins have found to be correlated with development of T2DM such as dioxins, cadmium, mercury and arsenic. Although the association is less established but it is found to cause beta cell dysfunction (Meo et al., 2015).

## 1.3. CRITERIA FOR DIABETES DIAGNOSIS:

For analyzing the severity and progression of diabetes the most primal task is diagnosis. Certain instructions for diabetes diagnosis and screening have been given by World Health Organization (WHO) and American Diabetes Association (ADA). ADA has set the limit for tagging a person with diabetes. Different tests used for diagnosis of T2DM are as follow:

### 1.3.1. Blood glucose test:

It shows that a reading of fasting glucose plasma (FGP) around and above 126 mg/dl should be diagnosed as diabetic. However, reading of random plasma glucose (RPG) if higher than 200mg/dl is diabetic and the postprandial glucose plasma which is after 2 hours of meal is referred as diabetic if more than 200mg/dl. Nonetheless, the range set for gestational diabetes is different. The reading if higher than 95mg/dl before meal and 140mg/dl after meal shows that gestational diabetes has set in. (Riddle, 2017). Moreover, the systems of diabetes encompass polydipsia (excessive thirsty feeling), polyphagia (binge eating), polyuria (extreme urine excretion), weight loss (Thurtell & Mackie, 2018).

### 1.3.2. OGTT (Oral Glucose Tolerance Test):

It tracks record of blood glucose level at intervals of 2 hours and this tracking should be for at least 4-6 hours after taking some glucose rich drink or oral ingestion of sugary drink. It basically provides information about the capacity of one's body to absorb glucose. If the measurement is higher than 200mg/dl it shows presence of diabetes (Punthakee, Goldenberg, Katz, & Committee, 2018).

### 1.3.3. Hemoglobin A1C:

Glycated Haemoglobin (HbA1c) is a sort of hemoglobin that attach to glucose molecule. It is formed when hemoglobin in red blood cells encounter glucose in glycation pathway and bind to it. This test is performed to check glucose level of last 3-4 months. The reason of this 3 months span is that the total life of RBCs is four months. Diabetic patients have a higher proportion of HbA1c than normal person and reveal the poorer control of blood glucose concentration. If the level of HbA1c is between 6 to 6.4 percent it indicate the propensity of developing diabetes and if more than 6.5 percent it infers diabetes presence in body (Duff et al., 2018). Moreover, in HbA1c a spike in number of free radicals is observed in RBCs. These reactive free radicals are dangerous for cell integrity and causes accumulation of blood cells and hamper blood flow (Nazish et al., 2018).

## 1.4. TREATMENTS FOR T2DM:

### 1.4.1. Diabetes management through lifestyle changes:

Diabetes is a multifactorial and heterogenous disease induced by both genetic n environmental factors. One of the prerequisite for controlling diabetes is through proper disease management plans which require help from a panel of endocrinologists, nutritionists, ophthalmologists. Since the disease progresses with aging, unhealthy food patterns, obesity and lack of physical activity and smoking and these have an adverse effect on insulin responses. A weight loss of around 10 pound than the original seems to generate a positive effect on insulin sensitivity by reducing glucose level. (Jacobs-van der Bruggen et al., 2009). Similarly, the diet plan of diabetic patients comprise

reduction of sugary, carbohydrate rich trans saturated foods and inclusion of more fibers containing nutrient rich vegetables and fruits. Likewise, addition of proper

aerobic exercises aids in maintaining body mass index (BMI) around 26kg/m2 and control hyperglycemic conditions. However, aging cannot be reversed with any treatment it is an irreversible phenomena but through lifestyle management it can be delayed to a minor scale (Magkos, Yannakoulia, Chan, & Mantzoros, 2009).

### 1.4.2. Insulin therapy:

Insulin analogues are now commercially available which are an instant source of providing artificially synthesized insulin in blood. These insulin are made by recombinant DNA technology through use of vector bacteria. (Kansagara, Fu, Freeman, Wolf, & Helfand, 2011). There are different sort of insulins and each has a distinct mode of action which slightly differ from each other to manage diabetes according to patients condition. They vary from rapid to short acting, intermediate acting insulins. NPH or isophane insulin, lente and ultralente which fall in intermediate category of insulin action can cause some asymmetrical assimilation and in some cases cause hyperglycemic condition. However, NovoLog, lispro and glulisine are widely used as rapid acting insulin and their action time falls in between 5-15 minutes (Kansagara et al., 2011).

### 1.4.3. Blood glucose monitoring:

Devices such as glucometer and CGM which is ***Continuous Glucose Monitoring*** are used to track down blood glucose readings. CGM is an approved device to track high and low level of blood glucose at regular intervals throughout the day (Mensinger et al., 2012).

### 1.4.4. Bariatric procedures:

The weight loss surgery is highly linked with diabetes remission, $HbA_1$ normal level and obesity control. In bariatric surgery the most famous is RYGBP (Rouxen Y gastric bypass) or gastric bypass surgery. This entail the size curtailment of stomach and around 5 to 10 pounds weight loss occur after every 7 days (Panunzi et al., 2016). They have an impact on GIP responses, insulin sensitivity, adipogenesis and glucose metabolism. However, demerits of these surgeries include excessive blood loss, hernia, dizziness, ulcers and other short and long term complications (Schauer et al., 2017).

### 1.4.5. Pharmaceutical Drugs as Medication:

**I.** **Glucophage**: commonly known as **metformin** was among one of the first authorized drug for T2DM treatment. It falls under the drug category of biguanids (Jackson et al., 1987). Metformin mode of action differs from others because it lessen glucose production from the hepatic cells and ameliorates insulin sensitivity and plays an active role in reducing glucose absorption in intestine. Hence, it is believed to somehow limit incidence of hypoglycemia in body (Pernicova & Korbonits, 2014).

**II.** **Meglitinides:** The efficacy of these drugs is shown because they aid the beta cells of pancreas to liberate insulin hormone. They have a distinct mechanism in comparison to sulfonylurea, they target ATP dependent K-channel for synthesis n release of insulin. These are termed as rapid action insulin drugs and they lessen hypoglycemia conditions of body (Gupta et al., 2016).

III. **Gliptins** also known as DPP-4 Inhibitors (Dipeptidyl peptidase-4 inhibitor) are a new alternative therapeutic drug for T2DM. DD4 inhibitor acts to hamper the activity of DPP4 enzyme. DPP4 enzyme acts in a way to dismantle GLP1 and GIP incretin hormones which aids in insulin production according to body's requirement so these inhibitors hamper the hyperglycemic condition by controlling DPP enzyme. (Hemmingsen, Sonne, Metzendorf, & Richter, 2017).

IV. **Thiazolidinediones (TZDs):** TZDs called known as glitazones are another therapeutic drug which is avidly used for T2DM treatment. Mode of action vary from others because TZDs bind with PPRG-gamma receptors. PPRG receptors are present in adipose tissues and they help in adipocyte differentiation and regulation of metabolism of fatty acids so glitazones basically aids in reducing body's resistance to insulin (Rizos, Kei, & Elisaf, 2016). However, there are some clinical risks associated with the use of TZD which include osteoporosis especially in women (Davidson, Mattison, Azoulay, & Krewski, 2018).

V. **SGLT2 Inhibitors:** Sodium glucose Cotransporter-2 (SGLT2) inhibitors play an instrumental role in treating T2DM by inhibiting the re absorbance of glucose by kidney hence by doing this they supplement glucose excretion phenomena and lower blood glucose level. These cotransporter proteins reside in the proximal region of kidney. the most common and high efficacy inhibitors are canagliflozin and dapagliflozin (Wu et al., 2016).

VI. **Sulfonylureas**: these drug act by invigorating insulin synthesis by pancreatic beta cells. They mechanism involve shutting down the K channel which has an ATP

receptor on it thus compelling the accumulation of ATPs in cell which in turn activate the Ca channel and cause exocytosis and insulin release. One of the best sulfonylureas are glipizide, glimepiride and glyburide. (Yu, Azoulay, Yin, Filion, & Suissa, 2018). However, there are some medical risks associated with the use of sulfonylureas such as hypoglycemic condition in patients taking this drug but the inexpensive and oral dosage advantage impels its consumption among patients (Riddle, 2017).

# *CHAPTER#2 LITERATURE REVIEW*

# 2. LITERATURE REVIEW

## 2.1. TRANSCRIPTION FACTOR 7 LIKE 2 (*TCF7L2*):

*TCF7L2* gene is basically a T cell specific transcription factor which encode protein coding genes. It is located on chromosome number 10 in humans and confer a dominant autosomal inheritance and its product is a transcription factor which plays a crucial role in protein coding product and any polymorphism in the genesis of transcription factor have deleterious repercussions and affect signaling pathways in which *TCF7L2* is involved (Pearson, 2009). *TCF7L2* gene belongs to the TCF family (Transcription factor family) or lymphoid enhancer factor (LEF) family which are a consortium of transcription factors which act by attaching to the high mobility group (HMG) box of DNA. HMG box is a chain of amino acids which bind to DNA and facilitates DNA specific binding to proteins and transcription factors (Štros, Launholt, & Grasser, 2007). *TCF7L2* gene exhibit it's expression in various tissues such as liver, beta cells of pancreas, gut, adipose tissues (Florez, 2007).

Hence *TCF7L2* can mediate bipartite functioning which involve both TCF and β-catenin. *TCF7L2* works along with beta catenin and facilitates insulin production in pancreatic beta cells. (Jin, 2016). Being a pleiotropic gene, it can exert its effect on various pathways including Wnt signaling pathway and other metabolic pathways. Moreover, *TCF7L2* can exert its influence by repressing or stimulating expression of gene (Sakhneny et al., 2018). This dual function is because of the alternative or differential splicing and interlinkage of various gene coregulators so *TCF7L2* has an opposite effect on beta cell. *TCF7L2* transcript is seen to cause a low production of protein product. The reduction of *TCF7L2* protein is linked with low level of GIP in beta cells. However, the downregulation of TCF protein is corelated with diminished beta cell function and could be a result of endoplasmic reticulum (ER) Stress that is induced by unfolded protein response (UPR) (Le Bacquer et al., 2011).

Moreover, the product of *TCF7L2* gene is present at substantially high and low amount in different tissues. The gene encoding transcript also varies in tissues. Also, isoforms of *TCF7L2* are generated because of the process of differential splicing (Le Bacquer et al., 2011). The various isoforms synthesized have the tendency to strive for attaching to the promoter region during the translation process and thus contributing in stimulation and suppression properties. Almost all the TCF family have the ability to use different transcription start sites (TSS) which impart this property of synthesizing functionally different protein. All the isoforms of *TCF7L2* contain the same binding sites but perform different functions. These isoforms have been grouped in categories depending on presence of C-terminus region (Helgason et al., 2007).

The whole exons present in *TCF7L2* are seventeen, out of these five have exhibited the capability of alternative splicing which are exons 4, 13, 14, 15 and 16. Exon 7 and 9 have shown the commonly used alternative splicing sites. Some infrequently used TSS are identified in exon 1. Some exons have shown similar sites but seen in greater number in different tissues such as exon 14 acts actively in adipose and myocytes while the exon 15 preponderate in lymphocytes. Thus, a complex splice site is generated with these alterations and variations in splice sites and give rise to a difference in protein expression level due to various protein isoforms (Osmark et al., 2009).

Moreover, different exons are involved in encoding and different products such as exon 10 and 11 of *TCF7L2* are implicated in attaching the HMG box of DNA binding. TLE/Groucho which are the corepressors of transcription binds specifically to the exon 9 of TF7L2. However, CBP (CREB binding protein) , p300 and CtBP (C-terminal binding protein) could only identify those isoforms which have long residues. CtBP which is basically a repressor halt *TCF7L2* expression and interact by binding to the sites encoded by exon 17 . similarly, CBP an activator requires long C terminus for attaching to the TCF isoform and grant the repressor and activator capabilities to the *TCF7L2* (Elfert et al., 2013).

## 2.2. ROLE OF *TCF7L2* IN WNT SIGNALING PATHWAY:

Wnt signaling is known to be indispensable for differentiation and proliferation of beta cells of pancreas and thus exert its influence on insulin production by alteration of GLP1 incretin hormone function. Wnt signaling is known to be instrumental in many pathophysiological roles and mostly importantly in embryonic development, cell adhesion, gene expression and in organ development (Moon, Kohn, De Ferrari, & Kaykas, 2004). The stem cell differentiation processes is an interchange of activated and inactivated state of wnt signaling pathway which require involvement of multiple proteins. In activating state, wnt signaling is necessary for muscle cells formation. Conversely, in repressing state factors such as *TCF7L2* binds with it and leads to adipocyte formation which have an adverse effect on insulin sensitivity and leads to T2DM and other heart diseases (Sharpe, Lawrence, & Arias, 2001).

Wnt signaling which is basically a signal transduction pathway include canonical and non canonical pathways. Human cells have nineteen sorts of Wnt isoforms which can show their functions through these canonical and noncanonical pathways. (Hansson, Zhou, Renström, & Osmark, 2010). Wnt are the growth stimulatory proteins. In canonical pathway, the effector molecules are TCF family and beta catenin (Huelsken & Behrens, 2002). Wnt signals are a group of signals that are stimulated after ligand receptor interaction.. There are around 10 receptors, eighteen ligands, however; despite multiple receptors wnt signaling is activated by a single mediator that is beta catenin. These wnt proteins are believed to be transported by the encapsulation in exosomes. Wnt pathway is highly conserved. Beta catenin is translocated through the receptor present in the plasma membrane to the cell nucleus. (Lecarpentier et al., 2017).

Beta catenin is regulated by action of degradation complex. The degradation complex contain a set of proteins which comprises of CK1 (casein kinase-1) , GSK3 (glycogen synthase kinase-3) , APC, Axin, Dvl (disheveled), β TrCP, (β-transducing repeat-containing protein), APC (Adenomatous polyposis coli) and phosphorylated ERK (Jho et al., 2002). All of them are attached

to beta catenin and leads to phosphorylation and ubiquitination of whole complex and eventually to the degradation of the whole complex by proteasome which is mediated through APC activity. In these, GSK3 plays the role of a negative modulator of catenin pathway. Hence, it keeps level of beta catenin low and this happens in inactivate state when there is no Wnt signal. (Barker, 2008). So in resting state in cytosol there is low concentration of beta catenin because it is controlled by degradation complex and TCF proteins (containing HMG box) action is halted by Groucho and CtBP (C terminal binding protein) which disable TCF to bind with DNA and conscript other nuclear corepressors such as histone deacetylases (HDACs) onto the promoters and suppress gene expression. Moreover, HMG box repressor (HBP1) which is a negative regulator hampers Wnt signaling pathway through connections with TCF family (Sampson et al., 2001).

Conversely, when there is wnt signal it leads to activated state of pathway and wnt transportation occur through exosome and Wnt molecule act like an ligand and attach to its receptor called Frizzeled. After ligand receptor interaction, LRP (lipoprotein receptor proteins) that is a coreceptor is phosphorylated (Moon et al., 2004). It facilitates the translocation of destruction complex to the LRP which is now not ubiquinated, intercept and block the phosphorylated conditional hence beta catenin degradation and there is an expansion of beta catenin concentration in cytosol. So beta catenin enters the nucleus and displace Groucho from TCF and itself attach to TCF gene so there is formation of bipartite TCF/beta catenin transcription factor which in return leads to transcription of Wnt target genes by enabling recruitment of cAMP protein binding proteins (CBP). This bipartite also make contact with Smad4 which is a coactivator of signal transduction pathway and a member of TGFβ receptors. (Nishita et al., 2000).

After the activation of WNT signal transduction pathway, the interaction of beta catenin and *TCF7L2* leads to the functioning of various target genes. Thus, the activated genes suppresses production of proglucogon. So, it is postulated that *TCF7L2* performs the role of effector in this pathway and manages glucose synthesis in liver and pancreatic cells (Welters & Kulkarni, 2008).

However, *TCF7L2* interacts with different cells and perform different functions in different organs. In liver, it causes negative regulation of gluconeogenesis by enhanced catenin/TCF activity (Ip, Shao, Chiang, & Jin, 2012). In pancreas, both *TCF7L2* and beta catenin work together in regulating insulin production. In intestine, this bipartite transcription factors are essential for GLP1 production which concomitantly plays role in insulin production. Likewise, this catenin/TCF complex activation enhances leptin production which plays a crucial role in energy balance and food consumption by transducing signals of satiety in brain and thus influencing body's requirement of food (Klok, Jakobsdottir, & Drent, 2007). Thus, TCF and catenin helps to regulate metabolic hormones in terms of food consumption and other changes. Usually GIP and insulin level rises after consumption of food and body's glucagon level decreases (Chiang, Ip, & Jin, 2012).

Moreover, *TCF7L2* is considered to play a fundamental role in regulating MYC expression. Wnt signaling is believed to enhance cell proliferation. The underlying reason of this is *TCF7L2* impact on targets like MYC and Cyclin D1. Myc are a group of regulator genes that encode transcription factors. *TCF7L2* attaches on the promoter side and function as a suppressor or activator depending on the presence or absence of CTNNB1 (Prokunina-Olsson, Kaplan, Schadt, & Collins, 2009).

Another noteworthy feature of wnt signaling is its impact on multiple target genes. The bipartite complex causes positive or negative regulation of signaling components such as Axin2, frizzle, LRP, Dvl. The repression of frizzle and LRP and activation of Axin causes a negative regulation. Conversely, positive regulation is seen while activating Rspo genes (Cadigan & Waterman, 2012).

## 2.3. ROLE OF *TCF7L2* IN PATHOGENESIS OF T2DM:

The gene product of *TCF7L2* is a high mobility complex HMG box (DNA binding domain) transcription factor which is involved in pathogenesis of diabetes mellitus. High expression of *TCF7L2* is observed in pancreas, brain, colon, lungs, intestine and alternate splice variants generated through alternative splicing results in *TCF7L2* gene product that either enhance or suppress Wnt signaling pathway (Florez et al., 2006). In studies it is believed that *TCF7L2* might have an influence on the ability of pancreatic cells to synthesize insulin by and adverse effect on GLP-1 and can lead to disrupted GLP-1 insulin production. In another study, it is postulated that *TCF7L2* can have a pleiotropic effect and any change could lead to development of T2D. (Prokunina-Olsson, Welch, et al., 2009).

*TCF7L2* acts as a regulator of glucose homeostasis through the glucagon gene so *TCF7L2* controls the activity of proglucagon gene which encode glucagon and incretin molecules. Proglucagon gene leads to the formation of glucagon by alpha cells of pancreas and the same proglucagon gene cause formation of incretin molecules in intestine and brain. Glucagon gene (GCG) is a prerequisite for encoding GLP1 incretin molecule in intestine.(Shao et al., 2013) GCG gene work by enhancing the gluconeogenesis or glucose formation and by lowering the glucose breakdown process or glycolysis (Schäfer et al., 2007). Glucagon is synthesized in alpha cells of pancreas Hence, *TCF7L2* modulates the synthesis of proglucagon and GLP1 in intestine.

Some studies have suggested high *TCF7L2* expression in diabetic patients but it doesn't lead to an equally high glucagon production level. In fact, some studies have reported that carriers of risk SNPs or heterozygous for SNPs are having a disruptive incretin insulin production in non diabetic patients, however, their incretin level falls in normal range when administered high level of glucose. Another study has reported direct involvement of *TCF7L2* in influencing beta cells of pancreas and thus insulin production and could enhance proliferation process of beta cells. Previously, T2DM was considered to occur because of decreased insulin activity; however,

recently, it is believed to have occurred because of less production of insulin by body. Hence, recent studies postulate that the diseased individuals have low insulin production because of many factors and beta cells are main targets of *TCF7L2* while was previously considered as an indirect result of *TCF7L2* activity. (Hoppler & Kavanagh, 2007).

In regards with association of pancreatic beta cells and *TCF7L2*, it is seen that dysregulation of glucose level in liver also leads to pathogenesis of T2DM. Liver is a major organ which is involved in glucose metabolism and glucose homeostasis by storing extra glucose in body in form of glycogen and breaking these stored glucose at the time of need in body by the process of gluconeogenesis. Studies have shown a link of *TCF7L2* gene and hepatic glucose metabolism. In normal conditions after food intake insulin is secreted by body which activate AKT pathway. (Huang, Liu, Guo, & Su, 2018)This signaling pathway lessen gluconeogenesis process in liver and enhances utilization of glucose by body. However, if body is experiencing insulin insensitivity, the AKT signaling pathways malfunctions and is unable to control HGP. The transcription factors other than *TCF7L2* important in regulation of HGP are FoxO1 and CREB (cAMP response element binding protein). (Oh, Han, Kim, & Koo, 2013).

However, a single polymorphism change in *TCF7L2* gene can cause an increased level of hepatic glucose production (HGP). The exact underlying mechanism of low insulin sensitivity and high HGP is not known but it is postulated that several enzymes and genes are involved in controlling hepatic glucose metabolism. Enzymes are encoded by genes and any alteration in expression of these genes leads to a disturbance and elevated level of HGP. These genes include *Pck1, G6pc, Fbp1*. (Zhang, Yang, Chen, & Su, 2018). Some studies have hypothesized the direct involvement of *TCF7L2* in regulating expression of these genes encoding enzymes and attachment of *TCF7L2* to several genes which are believed to play a crucial role in HGP such as glycogen synthase 2 (GS2), Pyruvate dehydrogenase kinase 4 (PDK4), Carnitine Palmitoyltransferase 1A (*CPT1A*), *Insulin receptor substrate 1* (*IRS-1*), Adiponectin receptor 1 (AdipoR1). Each of these genes are

incumbent for proper HGP such as GS2 is synthesized exclusively in liver and involved in glucose metabolism any change in this would lead to postprandial hyperglycaemia. (Norton et al., 2011). Thus, a reduced level or silencing of *TCF7L2* would cause an elevated level of blood.

## 2.4. SNPS OF *TCF7L2*:

Various studies have reported the strong linkage between *TCF7L2* and type 2 diabetes mellitus. *TCF7L2* associated haplotypes (group of alleles in an individual) are found to be located in the noncoding or intronic region of the gene. The polymorphism of *TCF7L2* that have a profound effect in disease pathogenesis is found to reside in intronic region of the gene (Saxena et al., 2006). As known the intronic region is spliced out in the process of translation having on apparent role in protein production. However, the recent studies have concluded that mutations in non coding regions are play a havoc for proteins such as could it can cause misfolding of proteins, alter splicing sites and splice junctions, can induce insertions and deletions, and ultimately dysregulation in protein functions. The location of where intronic single nucleotide mutation has occurred is very important its proximity to splice sites determine the ultimate destiny of protein and can affect the binding of transcription factors and enhancers thus affecting overall expression of gene (Osmark et al., 2009).

Several intronic SNPs of *TCF7L2* have been identifies which increases diabetes type 2 susceptibility in humans. The most well studied in this regard is rs 7903146 which was the first identified SNP of *TCF7L2* and it has been unequivocally declared the most prevalent and common SNP of *TCF7L2* with an extremely high detrimental effects and make body vulnerable to diabetes. (Wegner et al., 2008) According to the Genome wide association studies other mapped *TCF7L2* SNPS are s12255372, rs7901695, rs11196205, rs290487, and rs11196218 and rs7895340 which are found to be within linkage disequilibrium (LD) block of 92 kb. This well defined LD spans between intron 3 and intron 4 of *TCF7L2* out of the total 13 exons. These intronic variant somehow

alter the transcripts of mRNA and protein overall expression. Several studies have endeavored on explaining the reduced formation of mRNA transcripts in different body tissues e.g, myocytes, adipocytes, lymphoblastoid cells, hepatocytes (Nobrega, 2013). *TCF7L2* contains multiple variations of splicing which occur specifically between 3 and 4 exons around the linkage disequilibrium region, between 12 and 13 exons of C terminal and the alternative TSS. Hence, it can lead to production of transcripts which perform various functions from differentiation to proliferation (Peng et al., 2012). One study have reported the reduced expression of *TCF7L2* in patients suffering from obesity and their vulnerability to diabetes however it was cohort study. Still need to be done on large scale. Moreover, *TCF7L2* exons have the ability of alternative splicing and thus confers the body with a high molecular diversity but the underlying reason of tissue specific nature of *TCF7L2* splicing and its expression is not fully understand. The exons alternative slicing exhibit tissue specificity. These alternative splicing is distinctive in different tissues e.g, one form is exclusively for pancreas, other for liver e.t.c (Pang, Smith, & Humphries, 2013). SNPs can be present in both homozygous and heterozygous form. Individuals with heterozygous form carry both alleles one from each parent, however, if an individual is homozygous of the risk allele he is more susceptible to the disease diabetes in this case depending on the disease causing frequency of SNP as compare to the non carrier individuals. Two major portions of *TCF7L2* contain HMG DNA binding box and a beta catenin domain. Both of these by specified exons, out of which five are prominent in alternative splicing and gene span in 215 kb in chromosome (Q. Ren et al., 2008).

SNPS identified to be associated with *TCF7L2* according to NCBI are around 5501. HapMap website and SNP databases are widely used sites for screening tag SNPS. Most common tag SNPS for *TCF7L2* are rs7903146, *rs4506565* and *rs12255372*. These are obtained through linkage disequilibrium and minor allele frequency (MAF) studies. SNPS results are found to be different in different ethnic populations (J. Wang et al., 2013).

In *rs 12255372* polymorphism occur at 293 position at microsatellite region (DG10S478) and in this nucleotide G is being replaced with T. The nucleotide transition alters the whole translation process. In this G is the major allele or the ancestral allele while T is the minor allele or risk allele. A substitution fom G to T leads to increase the susceptibility to diabetes. In epidemiological studies different results are found due to involvement of various heterogeneity in progression of diabetes. Other factors such as age, gender also plays a role in diabetes susceptibility. European and Caucasian countries have a strong linkage disequilibrium is seen between these SNPS however in African countries weaker linkage disequilibrium and inconsistent results are reported. Inconsistency in SNP results is seen in different ethnic populations. To ascertain the mechanism and genetic involvement of these SNPS with diabetes require an elaborative study of these SNPS across the globe (Yao et al., 2015).

Experimental analysis has shown that a decrease in *TCF7L2* expression could cause an elevation in cell apoptotic process of pancreatic cells. The depletion of *TCF7L2* also lowers insulin production and proliferation and this trend is observed more in variants of *TCF7L2*. A research in which assay of exon 13 and exon 14 was done elaborates how a splicing of these generates a CRARF protein structure which is pivotal in turning on Wnt signaling pathway. The CRARF domain is one of the most conserved region found in TCF family. Likewise, assay analysis of exon 13 reveal the trend of tissue specific splicing in *TCF7L2* expression. Expression level is different in different tissues such as pancreatic islets, muscle, adipocytes, monocytes. Various studies have validated the same results after assaying same splicing sites. However, limitations associated with assaying is the diversity of human tissues and its varying expression in different tissues. The difficulty in localizing all the splicing sites, small sample size and related problems of false negative and false positive.

*rs4506565* is another important SNP of *TCF7L2* which is recently find to be associated with diabetes accounts for 90 percent as that of rs 7903146. *Rs 4506565* is located between exon 3 and

4 and in this the ancestral allele is T. However, single nucleotide polymorphism that takes place is the substitution of A with T. The minor allele frequency (MAF) count is 0.33. *rs 4506565* is found to be in strong linkage disequilibrium with rs 7903146 so rs7903146 could be a tag SNP for *rs 4506565*. However, other SNPS such as *rs 12255372* are not in strong LD in different populations and limited studies has been done on *rs 4506565* (Wirström, 2012) .

# ***CHAPTER#3 METHODOLOGY***

# 3. METHODOLOGY

## 3.1. STUDY SUBJECTS EVALUATION:

Study was designed in such a way to find out the rate of occurrence of T2DM with reference to *TCF7L2* gene SNPs (*rs12255372* and *rs4506565*) along with the healthy control samples to rule out the chances of error. For this, samples were collected from hospitals of Rawalpindi and Islamabad; PIMS hospital Islamabad, holy family hospital, military family hospital Rawalpindi.

Study subjects were divided into two types; diseased and control. Blood sample from 106 patients with T2DM and blood from 50 normal individuals were taken which was later used as control.

## 3.2. EXCLUSION AND INCLUSION CRITERIA:

### 3.2.1. Exclusion criteria:

Exclusion criteria involve certain traits or characteristics which disqualify a person from being included in a designed research. Patients suffering with diseases like HIV, AIDS, Hepatitis or other chronic conditions of heart, liver, kidney were excluded from the designed study. Moreover, Patients with, diagnosed with gestational diabetes, diabetes type 1, metabolic syndrome and mental disorders were also not included in study.

### 3.2.2. Inclusion criteria:

Patients suffering from type 2 diabetes mellitus, adult onset of diabetes were included in the study. Samples were taken from both genders; males and females and their age factor was also considered while allocating their groups. Patients fall in age range between 25 to 40 above.

## 3.3. DNA EXTRACTION METHODOLOGY:

### 3.3.1. Sample collection:

Ethical approval from Institutional Review Board (IRB) ASAB was required for carrying out blood sampling from T2DM patients. Informed consent from patients were also taken before extracting blood and put down in separate properly labelled tubes keeping in consideration the exclusion and inclusion criteria.

5ml blood was extracted from patient with help of phlebotomist and were kept in vacutainer. Vacutainer tubes which are sterilized tubes were labelled with patients name, age and ID. Samples were stored at 4˚C temperature in refrigerator and were placed on ice bags during transportation.

### 3.3.2. Extraction of DNA:

After sample collection, blood was transported in immunogenetics lab of Atta ur Rehman school of applied biosciences (ASAB) on cold packs. Cold packs are used in dispensing blood samples so as to maintain the blood temperature. Genomic DNA was extracted from the blood sample using phenol chloroform method .

### 3.3.3. Phenol chloroform method:

Phenol chloroform method is a widely known method for isolation and purification of DNA from blood sample. Extraction is a two day protocol. The process requires four solutions for proper separation of aqueous and organic layer and high yield of genomic DNA.

Day 1 protocol:

I. Shaking of EDTA tubes containing blood samples was done for 2-3 minutes. Afterwards, 750 ul of blood was taken out from EDTA tube with help of pipette and

transferred in an autoclaved centrifuge tube. Equal amount of solution A 750 ul was added into it.

II. Mix the solution by inverting the tubes 4-6 times and keep the tube at room temperature for about 5-10 minutes.

III. Centrifuge the solution on 13000 rpm for 1 minute in a microcentrifuge.

IV. Supernatant was discarded after centrifugation and the pellet was resuspended in 400 ul of solution A and this process was done for two times. Solution A containing Sucrose, Tris, Mgcl2 and Triton X are important for proper cell lysis

V. Again centrifugation was done at 13000 rpm for 1 minute.

VI. Supernatant was discarded and resuspended the pellet in 400ul of solution B along with 20ul of 20% SDS and 5ul of Proteinase K. SDS is a detergent which is important for solubilizing phosopholipids and denaturing of proteins and also for releasing cellular components while proteinase K inactivates nucleases and remove the remaining proteins.

VII. Vortex the solution a bit if pellet was not dissolved.

VIII. Afterwards, solution was incubated at 37˚C for a night.

**Day 2 protocol:**

I. The overnight incubated sample was taken out. Equal volume of solution C and solution D was added into a falcon tube. After proper mixing, 500ul of freshly prepared volume of solution C (phenol) and solution D (chloroform and isoamyl alcohol) was added in incubated sample. The purpose of adding phenol along with chloroform is to obtain a proper separate layer of aqueous solution and organic phases.

II. Centrifuge the sample for 10 mins at 13000 rpm in microcentrifuge.

III. After centrifugation, aqueous phase was taken out with help of micropipette in a new centrifuge tube.

IV. Again, equal volumes of solution D was added in aqueous phase. According to the aqueous phase taken out, solution D was added and then centrifuged at 13000rpm for mins.

V. After centrifugation, aqueous phase (the upper layer) was transferred again in a new tube and DNA precipitation was done by adding 55ul of sodium acetate 3M at pH 6 (used to dissolve cell membrane and to take out the DNA binding proteins) and equal quantity of Isopropanol that is 500ul and tube was inverted many times for DNA precipitation. Isopropanol should be chilled in order to get proper DNA thread.

VI. Centrifugation was done again at 13000 rpm for 10 mins and supernatant was discarded. Now to the DNA pellet 200ul of 100% ethanol was added and centrifuged at 13000 rpm for 7 mins and ethanol was discarded. This washing step was done twice. Purpose of ethanol washing to obtain pure form of DNA it will help in taking out of the DNA proteins from the solution.

VII. Second washing was done with 200ul of 70% ethanol and again centrifuged at 13000 rpm for 7 mins and later on ethanol was discarded.

VIII. Afterwards, the tube was inverted and DNA was dried for 30 mins at room temperature 37.

IX. After 30 mins, 200ul of TE buffer was added in precipitated DNA. The main purpose of adding TE buffer is to protect the DNA from any sort of degradation and to solubilize DNA.

| Solution A | Solution B | Solution C | Solution D |
|---|---|---|---|
| **0.32 M Sucrose** | 10Mm Tris (pH 7.5) | Phenol | Chloroform (24 volumes) |
| **10mM Tris (pH 7.5)** | 400mM Sodium chloride (Nacl) | | |
| **5mM Magnesium chloride (Mgcl2)** | | | Isoamyl alcohol (1 volume) |
| **Triton X -100** | | | |

Table 3.1. DNA extraction protocol

## 3.3.4. Gel electrophoresis:

Gel electrophoresis is the most widely used method for the separation and visualization of DNA fragments. For quantification of gnomic DNA, 1% agarose gel is prepared.

The method for agarose gel electrophoresis is as follow:

I. For gel preparation, 1g agarose powder was taken and by weighing balance quantity was measured.

II. This 1g agarose was added in 100ml of 1X TBE buffer and dissolved by microwaving if for about 3 minutes.

III. Once properly dissolved, let the solution cool and ethidium bromide 8µg/ml was added into mixture. Ethidium bromide is used for visualization because it’s an intercalating agent.

IV. Afterwards, mixture was poured in the casting tray and left for 15 minutes for proper solidification.

V. In gel tank, 1X TBE buffer was added and solidified agarose gel was place it. Extracted DNA was loaded in wells.

VI. Gel was run for 40 mins at 90 volts, 400 A current and analyzed in Gel doc (gel documentation system).

| Sr. no | Components | Quantity |
|---|---|---|
| **1.** | EDTA | 7.5/1000ml |
| **2.** | Boric acid | 55/1000ml |
| **3.** | Deionized water | 1000ml |
| **4.** | Tris base | 108/1000ml |

Table 3.2; 10X TBE buffer

### 3.3.5. Quantification through nanodrop

For quantification of genomic DNA, UV-Vis spectrophotometer or Thermo Scientific™ *NanoDrop* 2000 and 2000c were used to ascertain the purity or concentration of nucleic acids. For this, NF water was firstly used as a blank and then a drop of sample was added to measure absorbance of sample. Absorbance ratio 260/280 is used to determine absorbance measurement of sample and the wavelength at which a sample absorb ultraviolet light. A ratio approximately 1.8 is considered as pure DNA.

## 3.4. SINGLE NUCLEOTIDE POLYMORPHISM (SNP) DETECTION:

### 3.4.1. Designing of Allele specific primers:

For allele specific polymerase chain reaction (ASPCR) two forward and two reverse primers have been designed through softwares such as dbSNP, Ensembl, Oligocalc and UCSC genome browser manually.

In dbSNP, which is a public archive for finding variation in genes, SNP ID is entered and FASTA sequence of SNP is obtained from it which is then entered in Oligocalc (an online oligonucleotide calculator) and self complementarity and hairpin formation is checked. Afterwards, specific binding of primers through the target allele is checked through UCSC genome browser which is referred as in-silico PCR because it estimates theoretical results of PCR reaction and apprise about the amplicon size. Sets of forward and reverse primers are entered which are designed to amplify target DNA sequence and amplification is checked computationally.

*TCF7L2* gene SNP *rs12255372* is a G/T nucleotide substitution on human chromosome 10 and SNP *rs4506565* is a T/A single nucleotide polymorphism in human chromosome 10 of *TCF7L2* gene.

Table 3.3; Sequences of forward and reverse primer for ASPCR

| SR. no | Gene (*TCF7L2*) | Sequence | Melting Temp | Base pair (bp) | Product |
|---|---|---|---|---|---|
| **1.** | *rs 12255372* Forward FT | GGAATATCCAGGCAAGAAT | 56.3 | 19 bp | 354 |
| | *rs 12255372* Forward FG | GGAATATCCAGGCAAGAATG | 58.4 | 20bp | 214 |
| | *rs 12255372* Reverse RT | ACGCTTTGAAGGTAGAGAGG | 60.4 | 20bp | 354 |
| | *rs 12255372* Reverse RG | CTGAGATGTGAACACAAGCAG | 60.8 | 22bp | 214 |
| **2.** | *rs 4506565* Forward FT | TGGCGACCGAAGTGATAT | 57 | 18bp | 187 |
| | *rs 4506565* Forward FA | TGGCGACCGAAGTGATAA | 58 | 18bp | 325 |
| | *rs 4506565* Reverse RT | GACATGTTGCATCTCTCCATA | 56 | 21bp | 187 |
| | *rs 4506565* Reverse RA | GAGCACTGTGGCTTCCAT | 57 | 18bp | 325 |

### 3.4.2. Allele specific polymerase chain reaction ASPCR:

ASPCR (Allele specific polymerase chain reaction) is a technique that is now used for detection of single nucleotide polymorphism detection. It is quite useful in analyzing point mutations or any small deletion in human genome and identification of genetic disease. For this, oligonucleotide tetra primers were synthesized which contain a set of forward and reverse primers. These primers are used to amplify the particular portion of DNA carrying target SNP and analyze prevalence of a particular SNP in population. A total volume of 20µl reaction mixture was prepared containing forward and reverse primers in it. Preparation was done in laminar airflow hood. Afterwards, tubes were place in thermocycler 2720. PCR products that are obtained after reaction are analyzed with help of agarose gel electrophoresis.

Table 3.4; Profile of reaction mixture of *TCF7L2 rs 12255372* and *rs 4503535*

| **Components** | ***TCF7L2* rs 12255372** | ***TCF7L2* rs 4506565** |
|---|---|---|
| **Forward primers** | 1µl | 1µl |
| **Reverse primers** | 1µl | 1µl |
| **DNTPs 10Mm** | 2µl | 2µl |
| **MgCl2** | 2µl | 2.5µl |
| **Buffer** | 2µl | 2.5µl |
| **Thermus aquatics** | 0.25µl | 0.3µl |
| **PCR water** | 9.75µl | 9.8µl |
| **Genomic DNA** | 2µl | 2µl |

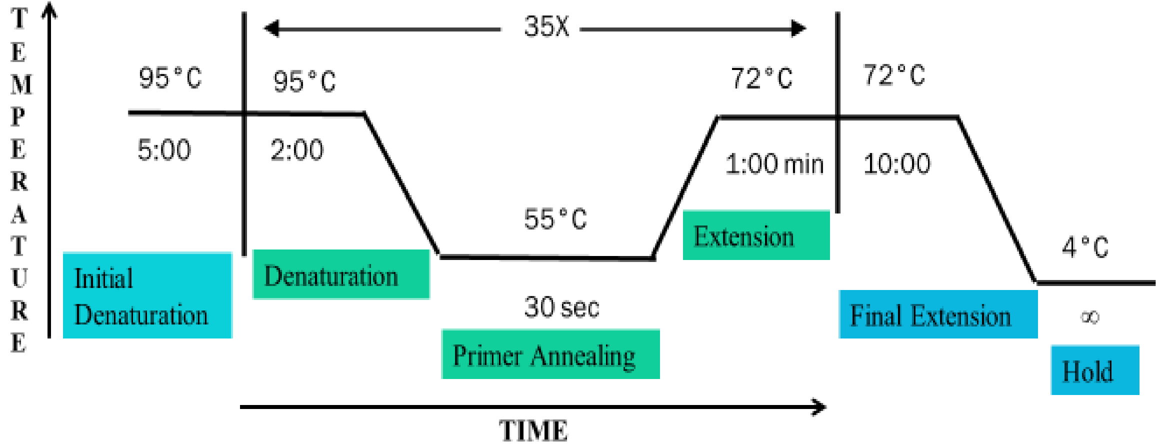


***Figure .3.1: PCR profile for TCF7L2 rs 12255372***

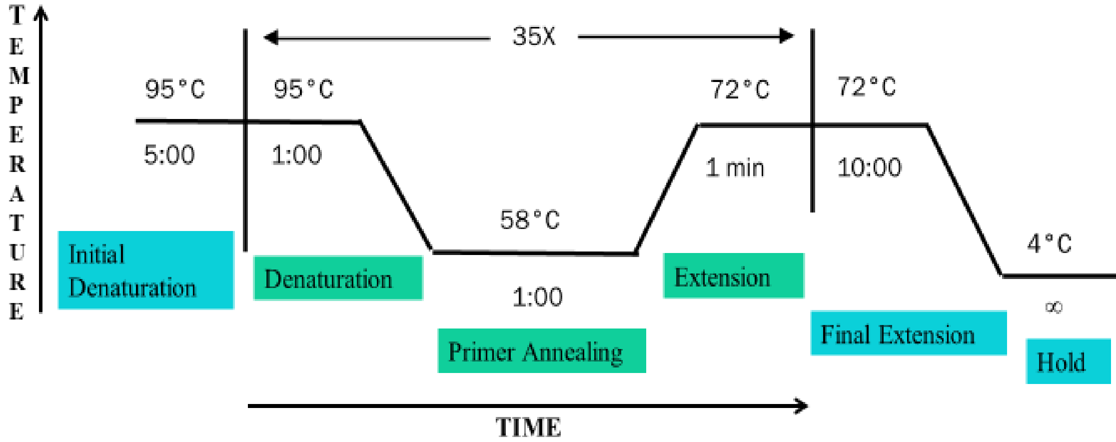


***Figure 3.2 : PCR profile for TCF7L2 rs 12255372***

### 3.4.3. Gel electrophoresis for PCR product:

After completion of PCR reaction, pcr products were analyzed in 2 percent agarose gel electrophoresis. For this, 2% w/v agarose was weighed and dissolved in 1X TBE buffer. After letting in cool for some time, 5µl ethidium bromide was added and poured in casting tray. After solidification of gel, loading dye was mixed with PCR products in ratio around 2:5. And loaded into the wells with micropipette. A 100bp ladder was also loaded in well for reference and analysis of bands.

## 3.4. Statistical analysis:

Statistical analysis was performed using different online tools such as 2x2 contingency table, Graphpad prism and Hardyweinberg equilibrium to ascertain allele frequency, odds ratio, relative risk, chi square and p value.

# *CHAPTER#4 RESULTS*

# 4. RESULTS:

Research was conducted to ascertain the association analysis of genetic polymorphism in *TCF7L2* gene and for this blood samples were collected from patients of type 2 diabetes mellitus and healthy controls from hospitals of Islamabad and Rawalpindi. A total of 187 blood samples were collected out of which 106 was of T2DM patients and 81 were of healthy individuals.

## 4.1. GENOMIC DNA EXTRACTION:

Blood samples were collected by using DNA extraction technique. For extracting DNA, phenol chloroform technique was used which is a two day protocol. For quantification of DNA, nanodrop and Thermo Scientific spectrophotometer were used. 260/280 ratio was penned down to ascertain purification of genomic DNA.

## 4.2. GENOTYPING OF *TCF7L2 rs 12255372* and *rs 4506565*:

Allele specific polymerase chain reaction has been performed on *TCF7L2 rs12255372* and *rs4506565*. Screening has been done for all the blood samples to figure out presence and absence of a particular allele. After amplification of DNA by allele specific PCR, the PCR product is visualized through agarose gel electrophoresis.

*TCF7L2 rs 12255372* is an intronic SNP variant which is located on the region of chromosome . In this, SNV (Single nucleotide variation) of G>T occur where G is the ancestral allele while T is the risk allele. Genotypic frequency for T2DM and control is given in figure while the allelic frequency of control vs experimental is depicted in graph in figure

However, electropherogram of 2 percent agarose Gel stained with ethidium bromide for visualization is given in figure and for both SNPs i.e. *TCF7L2 rs 12255372* and *TCF7L2 rs 4506565*.

*TCF7L2 rs 4506565* is located between exon 4 and exon 5 at chromosome 10q11.29 position. It contains A>T single nucleotide variation in which T is the ancestral allele. The allelic frequency

of control vs experimental is shown in figure while the genotypic frequency graph of  is shown in figure

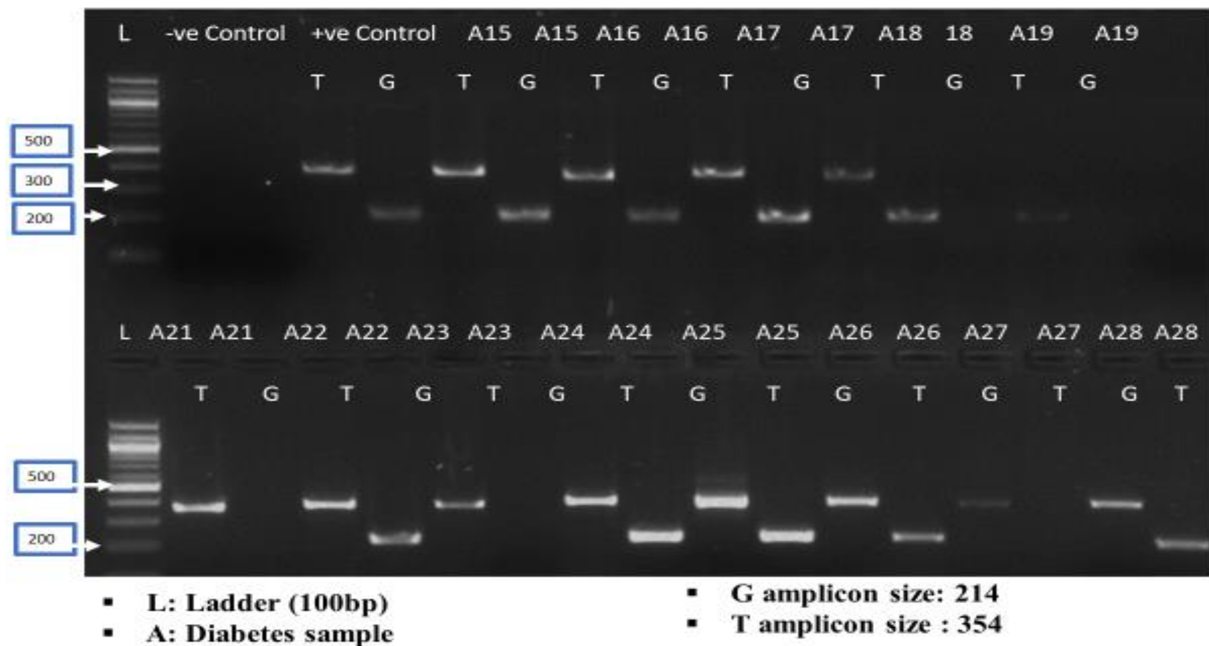


***Figure 4.1: TCF7L2 rs12255372 electropherogram of PCR products on the 2% agarose gel electrophoresis visualized on gel doc.***

The amplified PCR product of *TCF7L2 rs 12255372* was visualized on 2 percent agarose gel electrophoresis. The bands are seen under gel doc which makes them visible by observing them in ultraviolet light. The products are run along 100bp ladder to identify the size of amplified product and positive and negative control are also run for proper comparison.  The amplicon size of T allele shown in the first band which is a risk allele is 384bp while the second band is indicative of G allele whose amplicon size is 214bp. G allele is ancestral allele which has a protective effect in disease. The letter A indicates the disease blood samples, L is designated for ladder and C for control.

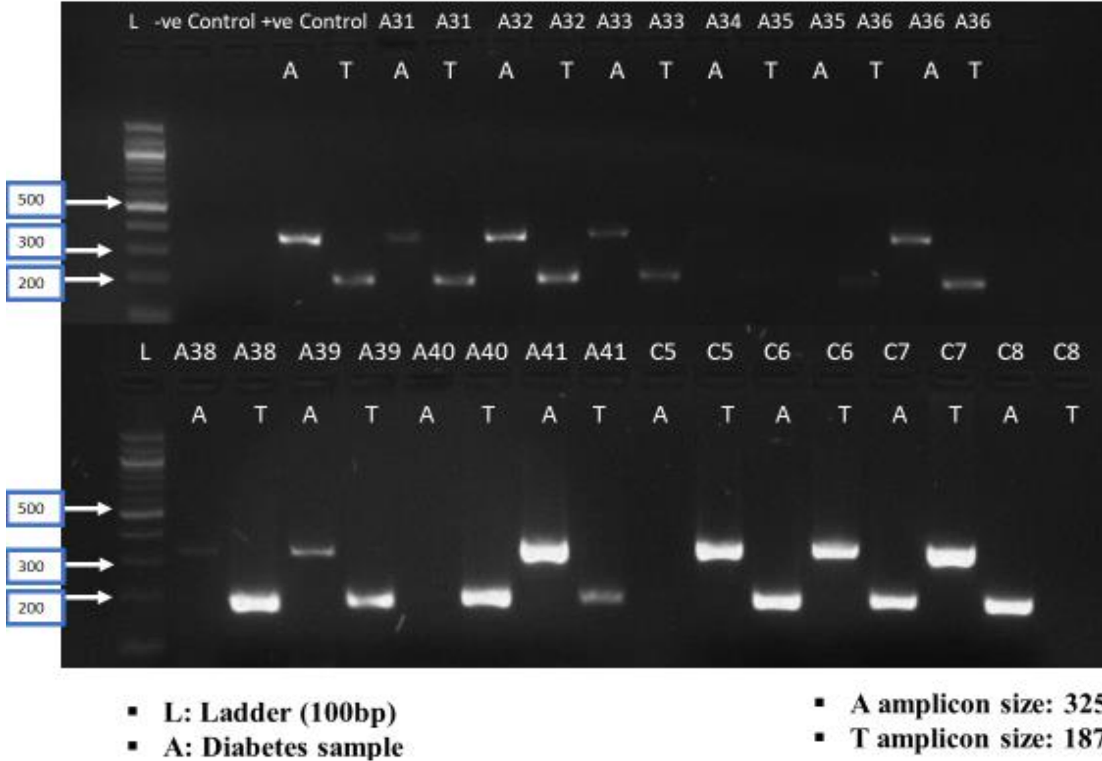


***Figure 4.2: TCF7L2 rs 4506565 electropherogram of amplified PCR products on the 2% agarose gel electrophoresis visualized on gel doc***

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The PCR products of *TCF7L2 rs4506565* are run along 100bp ladder, a negative control and positive control. The first band is of A allele which has a protective effect in T2DM and has an amplicon size of 325 while the second band is of T allele which is a risk allele in *rs 4506565* and has a size of 187bp and these bands are stained with ethidium bromide on gel.

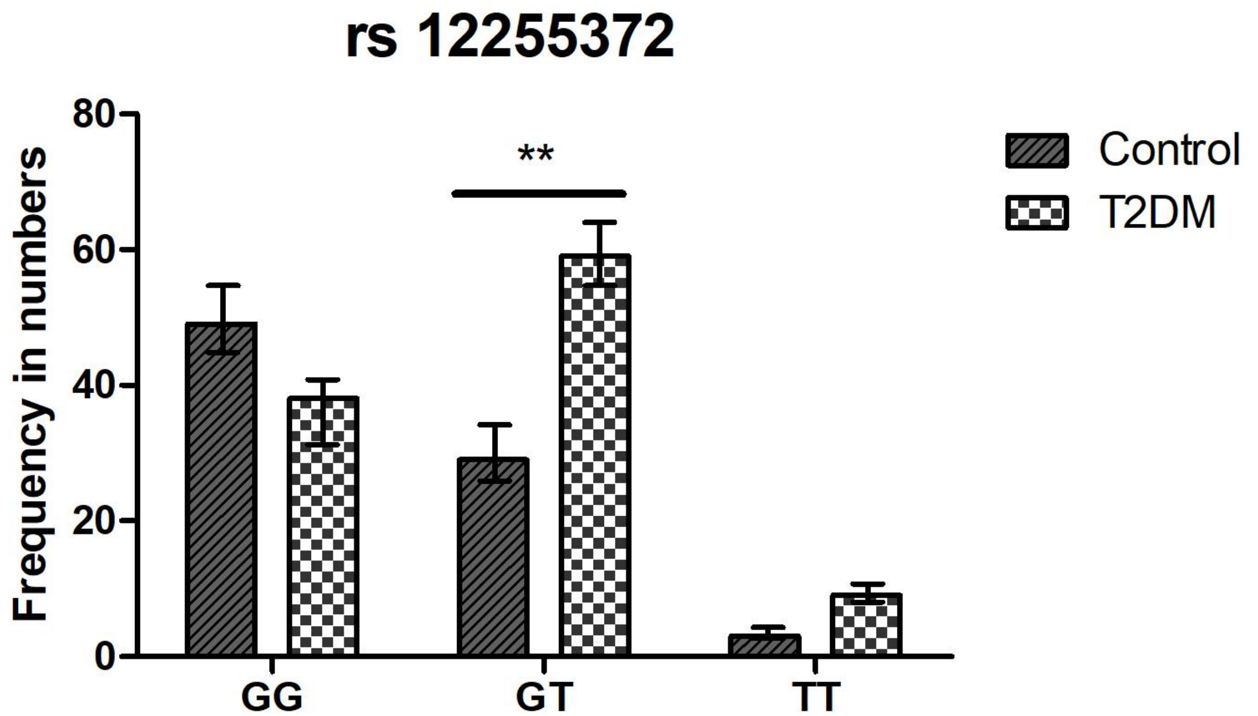


***Figure 4.3: Genotypic distribution of TCF7L2 rs 12255372 polymorphism in control vs experimental groups***

The genotypic distribution graphs of *TCF7L2 rs12255372* exhibit that the trend of heterozygous GT genotype is more frequently seen in T2DM than in control while the other genotype GG is prevalent in both control and T2DM however more common in controls. The highest trend of GT being seen in T2DM.

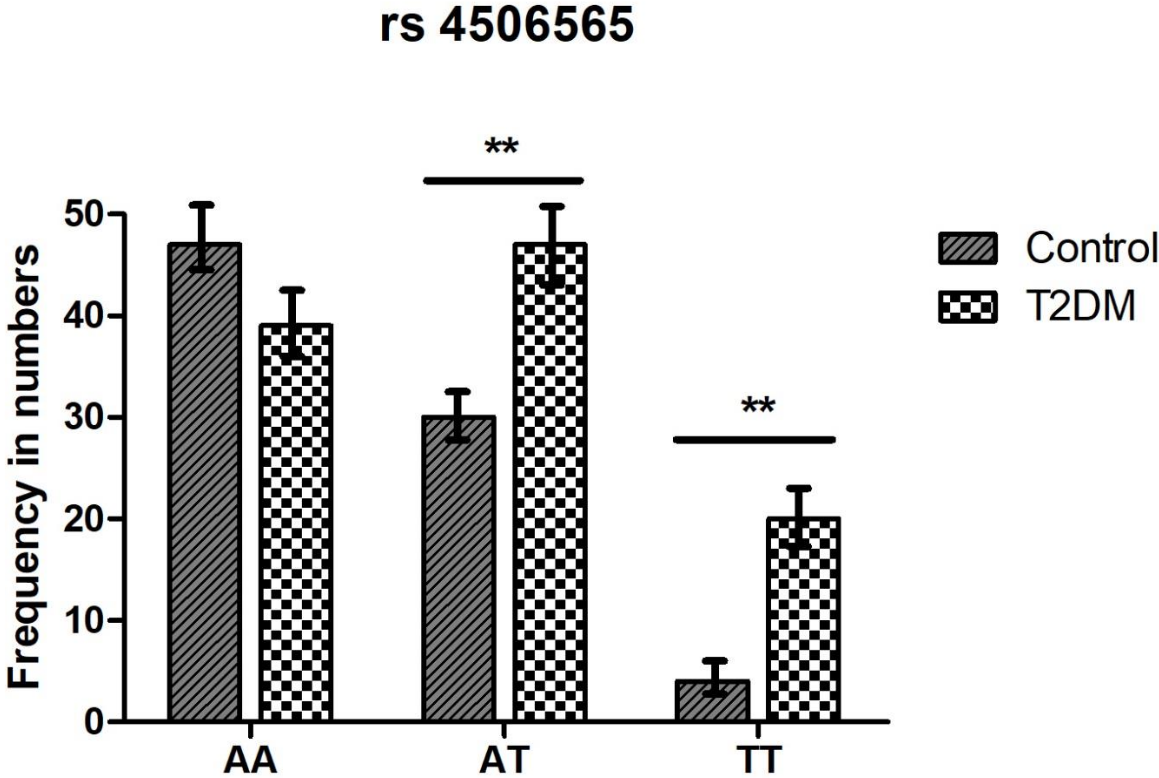


***Figure 4.4: Genotypic distribution of TCF7L2 rs 4506565 polymorphism in control vs experimental groups***

The genotypic distribution graphs of *TCF7L2 rs 4506565* exhibit that the trend of heterozygous AT genotype and homozygous TT more in T2DM than in control while the other genotype AA is prevalent in both control and T2DM however more common in controls. The highest trend of AT and TT being seen in T2DM.

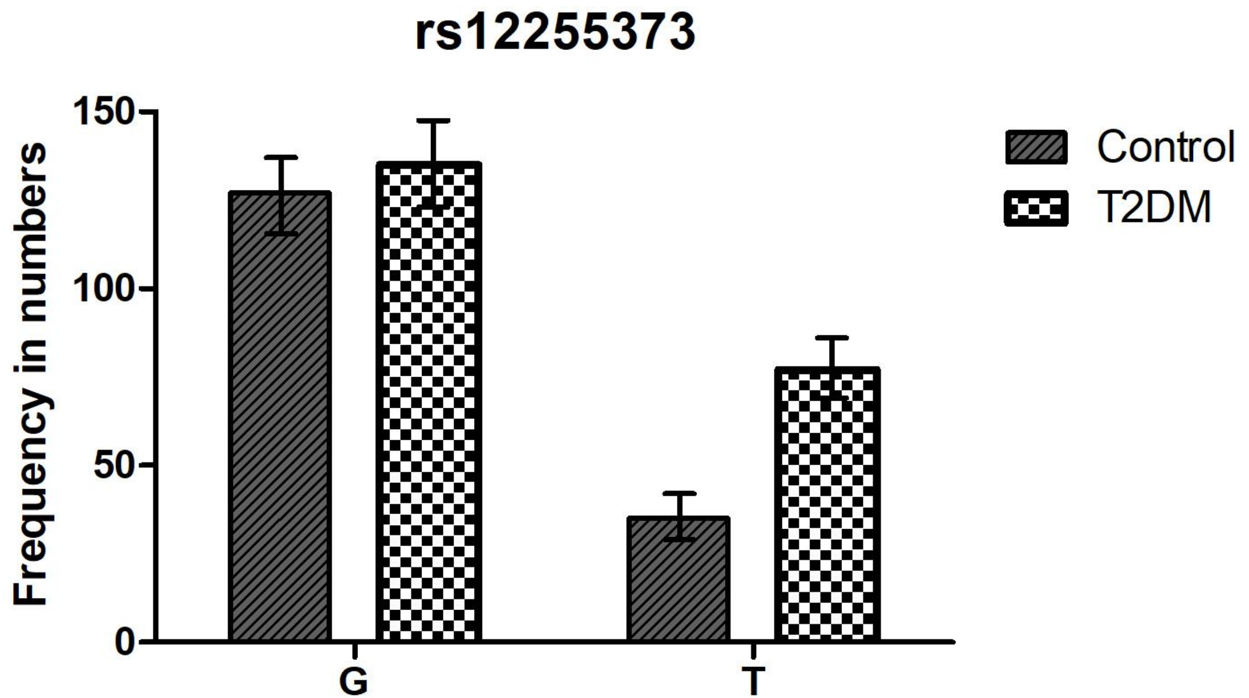


***Figure 4.5: Allelic distribution of TCF7L2 rs 12255372 polymorphism in control vs experimental groups***

The allelic frequency graph display the G and T allele frequency for *TCF7L2 rs 12255372* and exhibits the major allele frequency and minor allele frequency and indicates their distribution in cases and control.

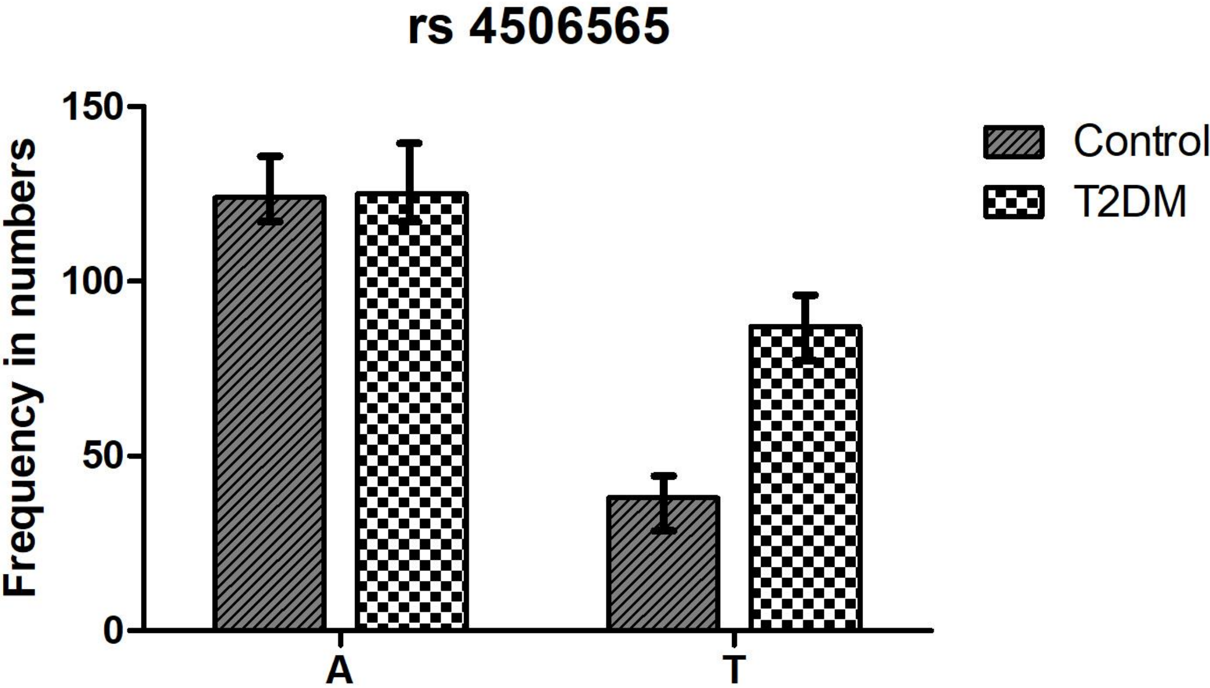


***Figure 4.6: Allelic distribution of TCF7L2 rs 4506565 polymorphism in control vs experimental groups***

The allelic frequency graph exhibits the A and T allele frequency for *TCF7L2 rs 4506565* and display the major allele frequency and minor allele frequency and indicates their distribution in control and T2DM.

**Table 4.1: Genotype and allele frequencies of *TCF7L2* rs 12255372 and rs 4506565 polymorphisms**

| SNP | Control | T2DM | Control vs T2DM | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Number (%) | Number (%) | OR (95%CI) | RR (95%CI) | P Value (HWE) | Chi square (HWE) | Chi square | P value |
| *rs 12255372* | | | | | | | | |
| Genotype | | | | | | | | |
| GG | 49 (60%) | 38 (35%) | 0.36 (0.4-0.8) | 0.59 (0.4-0.8) | 0.0006 | 11.21 | 11.48 | 0.003 |
| GT | 29 (36.3%) | 59 (56%) | 2.25 (1.2-4.07) | 1.55 (1.1-2.17) | 0.005 | 7.27 | | |
| TT | 3 (3.7%) | 9 (8.5%) | 2.41 (0.6-9.2) | 2.29 (0.6-8.17) | 0.153 | 1.75 | | |
| Allele | | | | | | | | |
| G | 127 (78.3%) | 135 (63.6%) | 0.48 (0.3-0.7) | 0.88 (0.7-0.9) | 0.001 | 9.48 | 9.74 | 0.0021 |
| T | 35 (21%) | 77 (36.4%) | 2.03 (1.1-2.4) | 1.66 (1.18-2.3) | 0.001 | 9.1 | | |
| *rs 4506565* | | | | | | | | |
| Genotype | | | | | | | | |
| AA | 47 (58%) | 39 (36%) | 0.4 (0.23-0.7) | 0.63 (0.46-0.86) | 0.003 | 8.3 | 12.04 | 0.0024 |
| AT | 30 (37%) | 47 (45%) | 1.35 (0.7-2.4) | 1.19 (0.83-1.7) | 0.19 | 1.1 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TT | 4 (5%) | 20 (19%) | 4.47 (1.4-13.6) | 3.8 (1.3-10) | 0.003 | 7.9 | | |
| Allele | | | | | | | | |
| A | 124 (76.5%) | 125 (59%) | 0.44 (0.27-0.6) | 0.77 (0.66-0.88) | 0.0002 | 11.98 | 12.76 | 0.004 |
| T | 38 (23.5%) | 87 (41%) | 2.27 (1.4-3.5) | 1.75 (1.2-2.4) | 0.0002 | 12.7 | | |

OR= Odd Ratio
RR= Relative Risk
95% CI, confidence interval
HWE: Hardy Weinberg Equilibrium
chi square x2-test and Fisher-exact test for genotype and for allele frequencies
Controls: 81
T2DM: 106

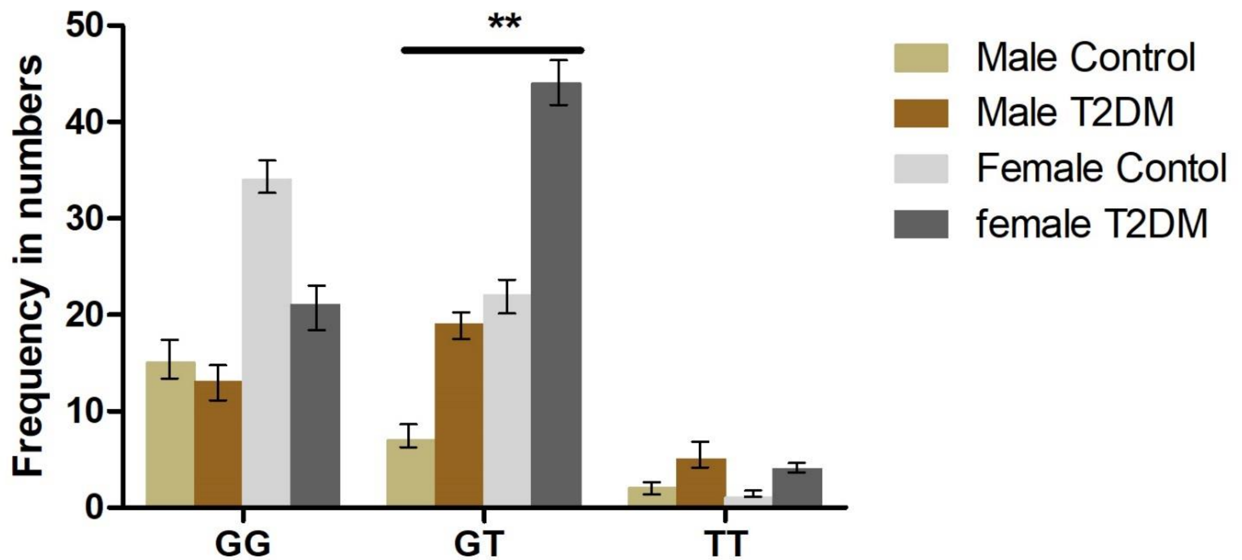


***Figure 4.7: Gender Based Genotypic Frequency of rs 12255372 Polymorphism in Control vs Experimental groups***

The results of comparison of Genotypic frequency of male and female control and experimental group exhibits a higher trend of heterozygous GT in T2DM patients than in control. The stark difference in their prevalence strongly link association of the heterozygous GT form with diabetes while the other distribution pattern of other two genotype is not significant. The statistical values of chi sqauare, df is 19.79,6 and P value is 0.0030**.

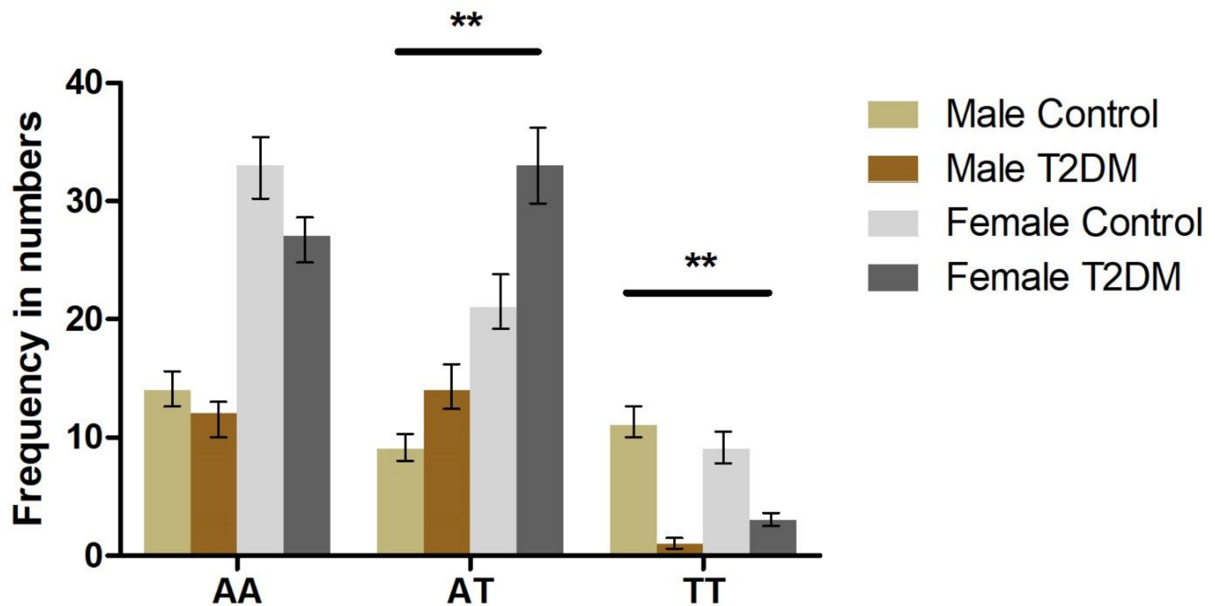


***Figure 4.8: Gender Based Genotypic Frequency of rs 4506565 Polymorphism in Male and Female Control vs Experimental groups***

The results of Genotypic frequency of control and experimental group in male female displays a higher trend of heterozygous AT in T2DM patients of female than in male and homozygous TT difference is significant in both male and female samples. The evident difference in their prevalence strongly link association with T2DM while the distribution pattern of homozygous genotype AA is almost similar and hence not significant. The statistical values of chi sqauare, df is 21.42,6 and P value is 0.0015**.

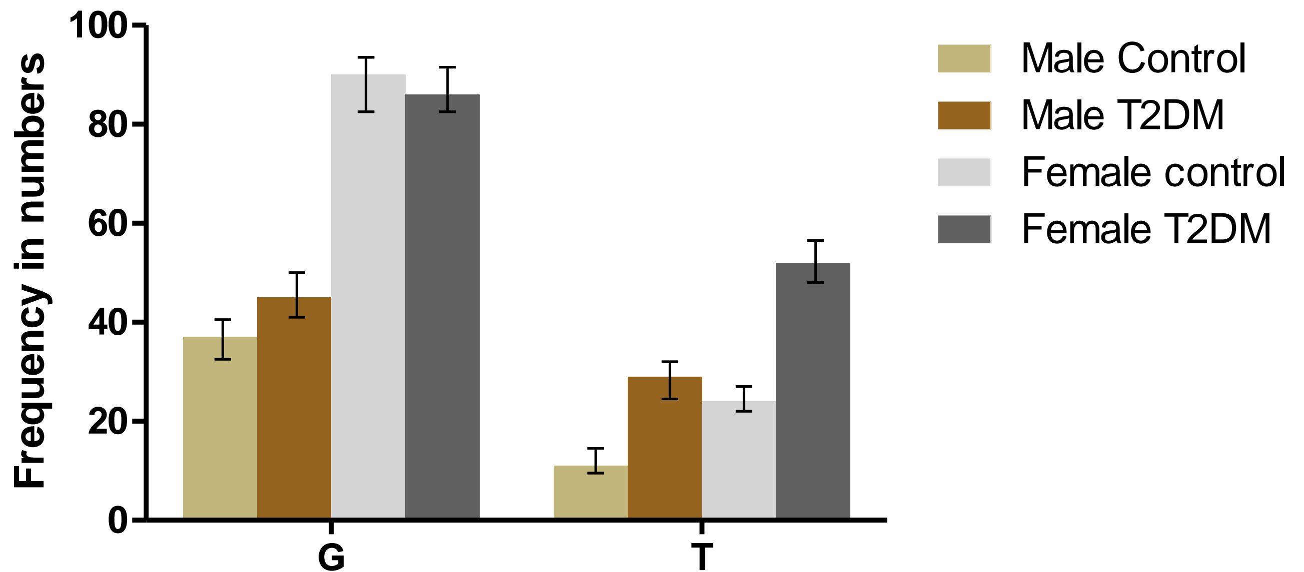


***Figure 4.9: Gender Based Allelic Frequency of rs 12255372 in control vs experimental groups***

The Allelic frequency graph of male and female control and T2DM shows the distribution of G and T allele in male and female population. No significant difference is seen in distribution pattern of G allele while the T allele is seen to be slightly higher in male and female T2DM than in male and female control. The value of chi square,df of allelic frequency is 11.94,3 and P value is 0.007.

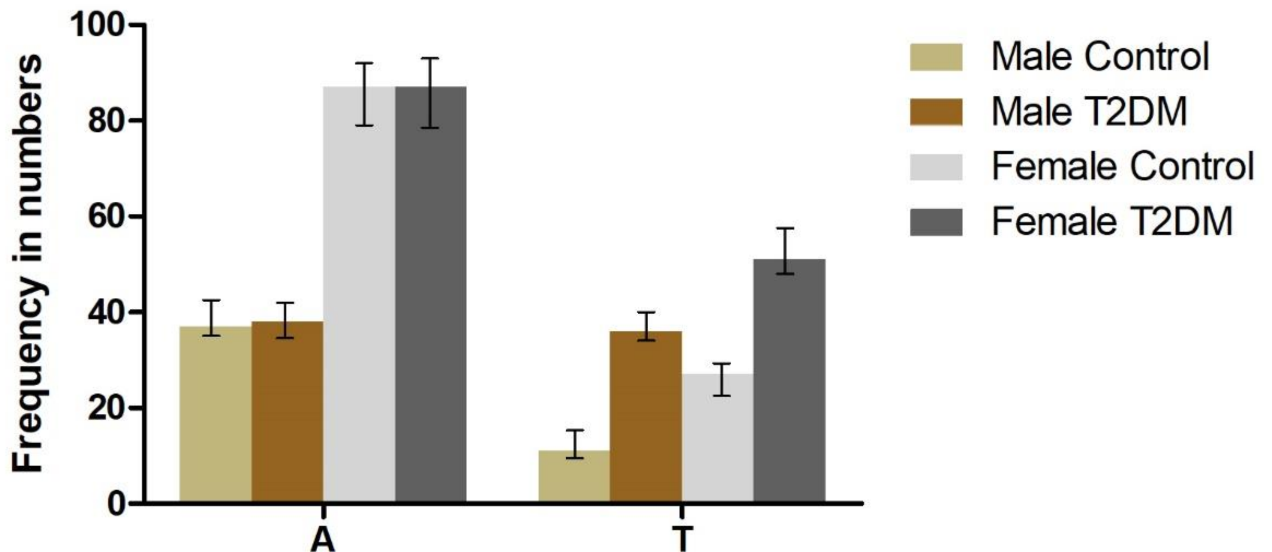


***Figure 4.10: Gender Based Allelic Frequency of rs4506565 Polymorphism in Male and Female Control vs Experimental groups***

The Allelic frequency graph of both genders of control and T2DM displays the distribution pattern of A and T allele in males and females. No substantial difference is observed in distribution pattern of A allele in male and female control vs T2DM groups and distribution is almost equal however risk T allele is seen to be slightly higher in male and female T2DM than in male and female control. The chi square,df value of allelic frequency is 15.72,3 and P value is 0.0013**.

**Table 4.2: Gender Based Genotypic and Allelic Frequencies of healthy controls and experimental groups along chi square and P value**

| Genotype | Male Control<br>n = 24 | Male T2DM<br>n =37 | Female Control<br>n = 57 | Female T2DM<br>n = 69 | Chi square,df | P value |
|---|---|---|---|---|---|---|
| *rs 12255372* | | | | | | |
| Genotype | | | | | | |
| GG | **15 (62.5%)** | **13 (35%)** | **34 (59.6%)** | **21 (30.4%)** | **19.79,6** | **0.0030** |
| GT | **7 (29.2%)** | **19 (51.3%)** | **22 (38.5%)** | **44 (63.7%)** | | |
| TT | **2 (8.3%)** | **5 (13.5%)** | **1 (1.7%)** | **4 (5.7%)** | | |
| Allele | | | | | | |
| G | **37 (77.8%)** | **45 (60.8%)** | **90 (79%)** | **86 (62.3%)** | **11.94,3** | **0.0076** |
| T | **11 (22.9%)** | **29 (39%)** | **24 (21%)** | **52 (37.6%)** | | |
| *rs 4506565* | | | | | | |
| Genotype | | | | | | |
| AA | **14 (58.3%)** | **12 (32.4%)** | **33 (57.8%)** | **27 (39%)** | **21.42,6** | **0.0015** |
| AT | **9 (37.5%)** | **14 (37.8%)** | **21 (36.8%)** | **33 (47.8%)** | | |
| TT | **1 (4.16%)** | **11 (29.7%)** | **3 (5.2%)** | **9 (13.4%)** | | |

| Allele | | | | | | |
|---|---|---|---|---|---|---|
| A | **37 (77%)** | **38 (51.3%)** | **87 (76.3%)** | **87 (63%)** | **15.72,3** | **0.0013** |
| T | **11 (23%)** | **36 (48.6%)** | **27 (23.6%)** | **51 (36.9%)** | | |

## 4.3. ASSOCIATION OF *TCF7L2* rs 12255372:

An extraordinary association is seen to be present between *TCF7L2 rs 12255372* and T2DM. The GT genotype specifies the risk of developing T2DM and the control vs T2DM statistical ratio with OR= 2.25, 95% CI= (1.2-4.07), P= 0.005** while the GG genotype exhibits a protective effect and its OR= 0.36, 95% CI= (0.4-0.8), P= 0.006. In contrast, the TT genotype has an OR= 2.41, 95% CI= (0.6-9.2), P= 0.153. The chi square (x2) value (11.48) with P= (< 0.003**) indicates a strong association between *rs12255372* with T2DM and the allelic frequency of G with OR= 0.48, 95% CI = (0.3-0.7), P= 0.001** and the allelic frequency of risk allele T with OR= 2.0, 95% CI= (1.1-2.4) P =).001** shows prevalence of alleles. The overall chi square (x2) value of allelic frequency (9.74) with P= (< 0.002**) suggests involvement of alleles in pathogenesis of T2DM.

## 4.4. ASSOCIATION OF *TCF7L2* rs 4506565:

A remarkable association is seen to be present between *TCF7L2 rs 4506565* and T2DM. The TT genotype specifies the risk of developing T2DM and the control vs T2DM statistical ratio with OR= 4.47, 95% CI= (1.4-13.6), P= 0.003** while the genotype AA exhibits a protective effect and its OR= 0.4, 95% CI= (0.23-0.7), P= 0.003. In contrast, the AT genotype has an OR= 1.35, 95% CI= (0.7-2.4), P= 0.19. The chi square (x2) value (12.04) with P= (< 0.0024**) suggests a substantial association between *rs4506565* with T2DM and the allelic frequency of A with OR= 0.44, 95% CI = (0.27-0.6), P= 0.002** and the allelic frequency of risk allele T with OR= 2.27, 95% CI= (1.4-3.5) P =0.002** shows prevalence of alleles. The overall chi square (x2) value of

allelic frequency (12.76) with P= (< 0.004**) suggests the involvement of alleles in pathogenesis of T2DM.

## 4.5. INSILICO ANALYSIS:

### 1. HAPLOREG V4.1 RESULTS:

| GENE | dbSNP ID | SNP location | Chromosome Position | Motifs changed by SNP | Enhancer Histone marks | GWAS hit |
|---|---|---|---|---|---|---|
| ***TCF7L2*** | *rs12255372* | Intron 5 | 10:113049143 | 5 Motifs altered | 19 tissues | 2 hits |
| ***TCF7L2*** | *rs4506565* | Intron 4 | 10.112996282 | 2 motifs altered | 4 tissues | N/A |

### 2. ASSP, ESE, Ensemble VEP Results:

| Gene | Alternative splice site predictor (ASSP) | Splice view | ESE finder (Exon Splicing Enhancer) | Ensemble VEP (Variant effect predictor) |
|---|---|---|---|---|
| ***TCF7L2*** **rs 12255372** | No change (proximity to 170 and 231 sites) | No change (proximity to 170 and 231 sites) | No change (Highest score for 156 and 351 position) | Risk factor |
| ***TCF7L2*** **rs 4506565** | No change (proximity to 37 and 110 sites) | No change (proximity to 37 and 110 sites) | No change (Highest score for 156 and 351 position) | N/A |

# ***CHAPTER#5 DISCUSSION***

# 5. DISCUSSION

Type 2 diabetes mellitus (T2DM) also termed as noninsulin dependent diabetes mellitus (NIDDM) is a condition in which there is a diminished or low production of insulin by the beta cells of pancreas or a reduced response of insulin thus leading to insulin resistance and accumulation of sugar in blood (X. Ren et al., 2018). Moreover, T2DM is known as multifactorial or heterogenous disorder because of the involvement of genetic as well as the environmental factors. Extracellular factors include age, obesity while the intracellular factors entail inflammation or an impairment immune response (Pickup, 2004). A strong association has been found with increased body weight which plays a prime role in onset of T2DM. Also, this deposition of body fat lead to several other health related issues such as cardiovascular problems, hypertension, dyslipidemia. (Ohashi, Shibata, Murohara, & Ouchi, 2014). However, the polygenic nature of T2DM makes it imperative to understand the action of various genes and how these genes increases the susceptibility to diabetes (Kaplan & Wagner, 2006). There are multiple genes who are believed to play a crucial role in progression of diabetes. Any mutation whether in exonic or intronic region of these genes affect the overall expression of protein product leading an impaired response of body towards insulin. (Ashcroft & Rorsman, 2004). An insight in the genetic polymorphism is important in this regard where a single nucleotide polymorphism can have a paramount impact on the protein outcome (Gloyn, 2003).

*TCF7L2* act in a tissue specific manner leading to its increased and decreased activity in various tissues. This tissue specific pattern is a major impediment in understanding the detailed role of its link with pathogenesis of T2DM (Bonnefond, Froguel, & Vaxillaire, 2010). The 17 exons in *TCF7L2* are distanced with intermittent introns. Five exons including exon 4, 13, 14, 15, 16 are involves in alternative splicing which can synthesize 13 different mRNA transcripts thus varying

expression of gene. The transcripts containing exon 1 and exon 2 are synthesized in equal numbers as that of transcripts which don't have exon 1 and exon 2. As known, exon 1 codes specifically for beta catenin so it is found that half of the transcripts don't contain beta catenin encoding exons. Resultantly, half of the *TCF7L2* isoforms produced don't have beta catenin encoding exons; however, the effects of such isoforms on gene regulation are poorly understood because they inhibit binding of *TCF7L2* isoforms to target *TCF7L2* gene (Weedon, 2007). The exon 14 and exon 15 are important because they aid to terminate reaction by introducing stop codon and both of these exons share similarity at mRNA and protein level. Moreover, exon14 is usually seen in adipose and muscle tissues while exon 15 is exclusively seen in islets. Though it is believed that most isoforms are deficient in synthesizing C terminal binding protein (CtBP) and which are inhibitors of WNT signaling pathway (Zhou et al., 2014). *TCF7L2* gene encode transcription factors that inhibit the proper functioning of Wnt signaling pathways However, studies have reported that intronic SNPS of *TCF7L2* cause predisposition to T2DM rather than the exonic coding region. Several articles have reported presence of a common microsatellite (DG10S478) region in *TCF7L2* gene and its correlation with T2DM however the exact mode of action of these intronic SNPS and their effect on gene expression is still uncertain. The need of elucidation is still there to understand how SNPS of non-coding region affect expression without having an obvious role in gene regulation. Research has been done on two SNPS *rs12255372* and *rs4506565* in this study of which *rs12255372* is found to be in strong LD with this microsatellite region. Various in silico tools have been used to ascertain the underlying cause of varying genetic expression of *TCF7L2* (Jian, Boerwinkle, & Liu, 2014).

The polymorphism found in *TCF7L2* SNPs has resulted in diminished insulin production specifically affecting the insulin production through GLP-1 indicating the faulty synthesis of insulin by intestine hormones. SNP *rs12255372* is located between intron 4 and 5 at chromosome

position 10:113049143 and *rs 4506565* is situated between intron 3 and 4 at chromosome position 10.112996282. The functional analysis is figured out using various in silico tools. Softwares such as ASSP, ESE, Splice View are used to ascertain the changes that occur in splice site because of polymorphism change. It is believed that both exonic and intronic sequences present can influence activity of silencers an enhancers. Exonic splice enhancers (ESE) and intronic splice enhances plays a crucial role in splicing. These sequences are identifies by five small ribonucleoproteins that form spliceosomes and bind to specific sequences present in exon and intron boundary (Yang et al., 2016). However, no change in splicing is observed because of these polymorphism substitution which is in accordance with the results of Pang el al article which stated that the pathogenicity of T2DM by variants of *TCF7L2* is not attributed to alternate splicing (Pang et al., 2013). The putative constitutive and cryptic splice sites predicted by these softwares lie at a distance from the polymorphism and no substantial difference is observed in splicing of exons. The putative splice sites of 170 and 231 site with highest scores in case of *rs 12255372* lie at a distance of around 25 nucleotides from the SNP change and in case of *rs4506565* sites which highest score 37 and 110 lie at a distance of 70 nucleotides and as mentioned in articles; for a SNP to be splice relevant it must be located in close proximity of splice site and a SNP splice relevance decreases as its distance from the splice site increase. SNP must be situated around 10 nucleotides from the splice site in order to have a functional effect on splicing (Faber, Glatting, Mueller, Risch, & Hotz-Wagenblatt, 2011).

Although the results of another online tool HaploReg v4.1 indicates the motifs changed by these SNPS of *TCF7L2*. HaploReg v4.1 is among one of the online available softwares for elucidating the alterations in functional properties of a gene by the non-coding region. It displays results in concordance with GWAS or any recent variants. The HaploReg results of *rs 12255372* and *rs 4506565* predicts the motif change caused by SNPS in *TCF7L2* gene. Motifs are considered

important in increasing the binding affinity of DNA binding protein. Motifs facilitates the binding to the region (Parker et al., 2013). Any change in motif could have adverse effects in gene transcription by impeding the regulatory effects. Two motifs (Gfi1, Gfi1b) are altered in case of SNP polymorphism at *rs 4506565* position while 5 motifs including (Cdc5 ,Nrf1, Pou2f2, Pou3f2, YY1) are altered if SNP polymorphism occur in *rs 12255372*. As known *TCF7L2* act in a tissue specific manner, repercussions of polymorphism could be different in different tissues.(Weise et al., 2009). The activity of *rs12255372* as enhancer is predicted in 19 tissues while only in 4 tissues in terms of *rs4506565*. This result is in line with result of Gaulton et al who mentioned that a plethora of open chromatin areas have been recognized in pancreatic cells containing regulatory elements (Groop, 2010). The risk allele substitution associated with T2DM in *TCF7L2* is instituted in open chromatin region. There are cis regulatory elements which are surrounded by free sites of chromatin (Pasquali et al., 2014). Moreover, there is a 92kb rich enhancer region found in *TCF7L2* gene region. Although researches has shown these enhancer rich regions vary individual to individual. However, whether the presence and interaction of multiple enhancer- transcriptional start sites TSS are inevitable for proper gene regulations remains ambiguous. Also, these interactions are believed to be redundant.(Ruiz-Narváez, 2014). The risk allele facilitates the attachment of DNA binding proteins thus perform an enhancer activity which leads to an increased level of transcription however in case of non risk allele no chromatin accessibility occur thus no attachment of DNA binding protein and thus a low level of transcription. (Ruiz-Narváez, 2014)

# CONCLUSION

TCF7LL2 SNPs *rs 12255372* and *rs4506565* represents a strong association with T2DM. Although there is a need to replicate these findings in larger number of samples and in other sections of population for validation. The genotypic distribution depicts higher prevalence of heterozygous trend in both SNPs. Likewise, the allelic distribution indicates a slighter higher trend in case of experimental than in control. Moreover, in silico softwares predict motif change as a result of this polymorphism; however no change in splicing is observed.

# FUTURE PROSPECTS

Study of *rs12255372* and *rs4506565* polymorphisms in *TCF7L2* gene of a relative sample size of T2DM patients in Pakistan can be helpful in providing much required information for the design of specific future novel therapeutic approaches, significantly focusing or targeting the population of T2DM individuals. Since Type 2 Diabetes is the forthcoming epidemic and over the past few decades the number of people with T2DM has grown exponentially globally. So there is a dire need to study their genetic association and risk factors to lessen the detrimental effects and to develop in-silico drugs targeting T2DM.

# *CHAPTER#6 REFRENCES*

# REFERENCES:


Aftab, H., Ambreen, A., Jamil, M., Garred, P., Petersen, J. H., Nielsen, S. D., . . . Christensen, D. L. (2017). High prevalence of diabetes and anthropometric heterogeneity among tuberculosis patients in Pakistan. *Tropical Medicine & International Health, 22*(4), 465-473.

Ashcroft, F. M., & Rorsman, P. (2004). Molecular defects in insulin secretion in type-2 diabetes. *Reviews in Endocrine and Metabolic Disorders, 5*(2), 135-142.

Asmat, U., Abad, K., & Ismail, K. (2016). Diabetes mellitus and oxidative stress—a concise review. *Saudi Pharmaceutical Journal, 24*(5), 547-553.

Baker, P., & Friel, S. (2014). Processed foods and the nutrition transition: evidence from A sia. *Obesity reviews, 15*(7), 564-577.

Baram, M., Atsmon-Raz, Y., Ma, B., Nussinov, R., & Miller, Y. (2016). Amylin–Aβ oligomers at atomic resolution using molecular dynamics simulations: a link between Type 2 diabetes and Alzheimer's disease. *Physical Chemistry Chemical Physics, 18*(4), 2330-2338.

Barker, N. (2008). The canonical Wnt/β-catenin signalling pathway *Wnt Signaling* (pp. 5-15): Springer.

Basit, A., Fawwad, A., Qureshi, H., & Shera, A. (2018). Prevalence of diabetes, pre-diabetes and associated risk factors: second National Diabetes Survey of Pakistan (NDSP), 2016–2017. *BMJ open, 8*(8), e020961.

Bonnefond, A., Froguel, P., & Vaxillaire, M. (2010). The emerging genetics of type 2 diabetes. *Trends in molecular medicine, 16*(9), 407-416.

Breidbart, E., Golden, L., Gonzaga-Jauregui, C., Deng, L., Lanzano, P., LeDuc, C., . . . Shuldiner, A. (2018). KCNJ11 MUTATION IN ONE FAMILY IS ASSOCIATED WITH ADULT-ONSET RATHER THAN NEONATAL-ONSET DIABETES MELLITUS. *AACE Clinical Case Reports, 4*(5), e411-e414.

Cadigan, K. M., & Waterman, M. L. (2012). TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harbor perspectives in biology*, a007906.

Cai, L., & Kang, Y. J. (2001). Oxidative stress and diabetic cardiomyopathy. *Cardiovascular toxicology, 1*(3), 181-193.

Chawla, A., Chawla, R., & Jaggi, S. (2016). Microvasular and macrovascular complications in diabetes mellitus: distinct or continuum? *Indian journal of endocrinology and metabolism, 20*(4), 546.

Chiang, Y.-t. A., Ip, W., & Jin, T. (2012). The role of the Wnt signaling pathway in incretin hormone production and function. *Frontiers in physiology, 3*, 273.

Conn, J. J., Jacobs, H. S., & Conway, G. S. (2000). The prevalence of polycystic ovaries in women with type 2 diabetes mellitus. *Clinical endocrinology, 52*(1), 81-86.

Cornelis, M. C., Zaitlen, N., Hu, F. B., Kraft, P., & Price, A. L. (2015). Genetic and environmental components of family history in type 2 diabetes. *Human genetics, 134*(2), 259-267.

Davidson, M. A., Mattison, D. R., Azoulay, L., & Krewski, D. (2018). Thiazolidinedione drugs in the treatment of type 2 diabetes mellitus: past, present and future. *Critical reviews in toxicology, 48*(1), 52-108.

Drucker, D. J., Habener, J. F., & Holst, J. J. (2017). Discovery, characterization, and clinical development of the glucagon-like peptides. *The Journal of clinical investigation, 127*(12), 4217-4227.

Duff, C. J., Solis-Trapala, I., Driskell, O. J., Holland, D., Wright, H., Waldron, J. L., . . . Hanna, F. W. (2018). The frequency of testing for glycated haemoglobin, HbA1c, is linked to the probability of achieving target levels in patients with suboptimally controlled diabetes mellitus. *Clinical Chemistry and Laboratory Medicine (CCLM), 57*(2), 296-304.

Elfert, S., Weise, A., Bruser, K., Biniossek, M. L., Jägle, S., Senghaas, N., & Hecht, A. (2013). Acetylation of human TCF4 (*TCF7L2*) proteins attenuates inhibition by the HBP1 repressor and induces a conformational change in the TCF4:: DNA complex. *PLoS One, 8*(4), e61867.

Erondu, N., Desai, M., Ways, K., & Meininger, G. (2015). Diabetic ketoacidosis and related events in the canagliflozin type 2 diabetes clinical program. *Diabetes care*, dc151251.

Faber, K., Glatting, K.-H., Mueller, P. J., Risch, A., & Hotz-Wagenblatt, A. (2011). Genome-wide prediction of splice-modifying SNPs in human genes using a new analysis pipeline called AASsites. *BMC bioinformatics, 12*(4), S2.

Florez, J. C. (2007). The new type 2 diabetes gene *TCF7L2*. *Current Opinion in Clinical Nutrition & Metabolic Care, 10*(4), 391-396.
Florez, J. C., Jablonski, K. A., Bayley, N., Pollin, T. I., de Bakker, P. I., Shuldiner, A. R., . . . Altshuler, D. (2006). *TCF7L2* polymorphisms and progression to diabetes in the Diabetes Prevention Program. *New England Journal of Medicine, 355*(3), 241-250.
Fuchsberger, C., Flannick, J., Teslovich, T. M., Mahajan, A., Agarwala, V., Gaulton, K. J., . . . McCarthy, D. J. (2016). The genetic architecture of type 2 diabetes. *Nature, 536*(7614), 41.
Gautier, J.-F., Choukem, S.-P., & Girard, J. (2008). Physiology of incretins (GIP and GLP-1) and abnormalities in type 2 diabetes. *Diabetes & metabolism, 34*, S65-S72.
Glovaci, D., Fan, W., & Wong, N. D. (2019). Epidemiology of Diabetes Mellitus and Cardiovascular Disease. *Current cardiology reports, 21*(4), 21.
Gloyn, A. L. (2003). The search for type 2 diabetes genes. *Ageing research reviews, 2*(2), 111-127.
Groop, L. (2010). Open chromatin and diabetes risk. *Nature genetics, 42*(3), 190.
Grytsay, V. (2017). Self-organization and fractality created by gluconeogenesis in the metabolic process. *arXiv preprint arXiv:1707.05678*.
Gupta, P., Bala, M., Gupta, S., Dua, A., Dabur, R., Injeti, E., & Mittal, A. (2016). Efficacy and risk profile of anti-diabetic therapies: Conventional vs traditional drugs—A mechanistic revisit to understand their mode of action. *Pharmacological research, 113*, 636-674.
Hajavi, J., Momtazi, A. A., Johnston, T. P., Banach, M., Majeed, M., & Sahebkar, A. (2017). Curcumin: A naturally occurring modulator of adipokines in diabetes. *Journal of cellular biochemistry, 118*(12), 4170-4182.
Hajiaghaalipour, F., Khalilpourfarshbafi, M., & Arya, A. (2015). Modulation of glucose transporter protein by dietary flavonoids in type 2 diabetes mellitus. *International journal of biological sciences, 11*(5), 508.
Hansson, O., Zhou, Y., Renström, E., & Osmark, P. (2010). Molecular function of *TCF7L2*: Consequences of *TCF7L2* splicing for molecular function and risk for type 2 diabetes. *Current diabetes reports, 10*(6), 444-451.
Hawley, N. L., & McGarvey, S. T. (2015). Obesity and diabetes in Pacific Islanders: the current burden and the need for urgent action. *Current diabetes reports, 15*(5), 29.
Helgason, A., Pálsson, S., Thorleifsson, G., Grant, S. F., Emilsson, V., Gunnarsdottir, S., . . . Reynisdottir, I. (2007). Refining the impact of *TCF7L2* gene variants on type 2 diabetes and adaptive evolution. *Nature genetics, 39*(2), 218.
Hemmingsen, B., Sonne, D. P., Metzendorf, M. I., & Richter, B. (2017). Dipeptidyl-peptidase (DPP)-4 inhibitors and glucagon-like peptide (GLP)-1 analogues for prevention or delay of type 2 diabetes mellitus and its associated complications in people at increased risk for the development of type 2 diabetes mellitus. *Cochrane Database of Systematic Reviews*(5).
Henriksen, E. J., Diamond-Stanic, M. K., & Marchionne, E. M. (2011). Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free Radical Biology and Medicine, 51*(5), 993-999.
Hjuler, S. T., Gydesen, S., Andreassen, K. V., Karsdal, M. A., & Henriksen, K. (2017). The dual amylin- and calcitonin-receptor agonist KBP-042 works as adjunct to metformin on fasting hyperglycemia and HbA1c in a rat model of type 2 diabetes. *Journal of Pharmacology and Experimental Therapeutics, 362*(1), 24-30.
Hoppler, S., & Kavanagh, C. L. (2007). Wnt signalling: variety at the core. *Journal of cell science, 120*(3), 385-393.
Horikoshi, M., Hara, K., Ito, C., Shojima, N., Nagai, R., Ueki, K., . . . Kadowaki, T. (2007). Variations in the HHEX gene are associated with increased risk of type 2 diabetes in the Japanese population. *Diabetologia, 50*(12), 2461-2466.
Huang, X., Liu, G., Guo, J., & Su, Z. (2018). The PI3K/AKT pathway in obesity and type 2 diabetes. *International journal of biological sciences, 14*(11), 1483.
Huelsken, J., & Behrens, J. (2002). The Wnt signalling pathway. *Journal of cell science, 115*(21), 3977-3978.
Ip, W., Shao, W., Chiang, Y.-t. A., & Jin, T. (2012). The Wnt signaling pathway effector *TCF7L2* is upregulated by insulin and represses hepatic gluconeogenesis. *American Journal of Physiology-Endocrinology and Metabolism, 303*(9), E1166-E1176.

Itariu, B. K., & Stulnig, T. M. (2014). Autoimmune aspects of type 2 diabetes mellitus-a mini-review. *Gerontology, 60*(3), 189-196.

Jackson, R. A., Hawa, M. I., Jaspan, J. B., Sim, B. M., DiSilvio, L., Featherbe, D., & Kurtz, A. B. (1987). Mechanism of metformin action in non-insulin-dependent diabetes. *Diabetes, 36*(5), 632-640.

Jacobs-van der Bruggen, M. A., van Baal, P. H., Hoogenveen, R. T., Feenstra, T. L., Briggs, A. H., Lawson, K., . . . Baan, C. A. (2009). Cost-effectiveness of lifestyle modification in diabetic patients. *Diabetes care, 32*(8), 1453-1458.

Jho, E.-h., Zhang, T., Domon, C., Joo, C.-K., Freund, J.-N., & Costantini, F. (2002). Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Molecular and cellular biology, 22*(4), 1172-1183.

Jian, X., Boerwinkle, E., & Liu, X. (2014). In silico tools for splicing defect prediction: a survey from the viewpoint of end users. *Genetics in Medicine, 16*(7), 497.

Jin, T. (2016). Current Knowledge on the Role of Wnt Signaling Pathway in Glucose Homeostasis *Molecular Nutrition and Diabetes* (pp. 357-369): Elsevier.

Kansagara, D., Fu, R., Freeman, M., Wolf, F., & Helfand, M. (2011). Intensive insulin therapy in hospitalized patients: a systematic review. *Annals of internal medicine, 154*(4), 268-282.

Kaplan, J. R., & Wagner, J. D. (2006). Type 2 diabetes—an introduction to the development and use of animal models. *ILAR journal, 47*(3), 181-185.

Kharroubi, A. T., & Darwish, H. M. (2015). Diabetes mellitus: The epidemic of the century. *World journal of diabetes, 6*(6), 850.

Klok, M., Jakobsdottir, S., & Drent, M. (2007). The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity reviews, 8*(1), 21-34.

Kohei, K. (2010). Pathophysiology of type 2 diabetes and its treatment policy. *JMAJ, 53*(1), 41-46.

Kriska, A., El Ghormli, L., Copeland, K. C., Higgins, J., Ievers-Landis, C. E., Levitt Katz, L. E., . . . Delahanty, L. M. (2018). Impact of lifestyle behavior change on glycemic control in youth with type 2 diabetes. *Pediatric diabetes, 19*(1), 36-44.

Kusminski, C. M., Bickel, P. E., & Scherer, P. E. (2016). Targeting adipose tissue in the treatment of obesity-associated diabetes. *Nature reviews Drug discovery, 15*(9), 639.

Lago, R. M., Singh, P. P., & Nesto, R. W. (2007). Diabetes and hypertension: Nature Publishing Group.

Lavin, D. P., White, M. F., & Brazil, D. P. (2016). IRS proteins and diabetic complications. *Diabetologia, 59*(11), 2280-2291.

Le Bacquer, O., Shu, L., Marchand, M., Neve, B., Paroni, F., Kerr Conte, J., . . . Maedler, K. (2011). *TCF7L2* splice variants have distinct effects on β-cell turnover and function. *Human molecular genetics, 20*(10), 1906-1915.

Lecarpentier, Y., Claes, V., Vallée, A., & Hébert, J.-L. (2017). Interactions between PPAR gamma and the canonical Wnt/beta-catenin pathway in type 2 diabetes and colon cancer. *PPAR research, 2017*.

Magkos, F., Yannakoulia, M., Chan, J. L., & Mantzoros, C. S. (2009). Management of the metabolic syndrome and type 2 diabetes through lifestyle modification. *Annual review of nutrition, 29*, 223-256.

Medici, F., Hawa, M., Ianari, A., Pyke, D., & Leslie, R. G. (1999). Concordance rate for type II diabetes mellitus in monozygotic twins: actuarial analysis. *Diabetologia, 42*(2), 146-150.

Mensinger, M. R., Dobbles, J. M., Kamath, A. U., Stadelmann, B., Ruppert, D. M., Salamati, N., & Yang, R. C. (2012). Systems and methods for blood glucose monitoring and alert delivery: Google Patents.

Meo, S., Memon, A., Sheikh, S., Rouq, F., Usmani, A. M., Hassan, A., & Arian, S. (2015). Effect of environmental air pollution on type 2 diabetes mellitus. *Eur Rev Med Pharmacol Sci, 19*(1), 123-128.

Moon, R. T., Kohn, A. D., De Ferrari, G. V., & Kaykas, A. (2004). WNT and β-catenin signalling: diseases and therapies. *Nature Reviews Genetics, 5*(9), 691.

Naaz, K., Kumar, A., & Choudhury, I. (2018). Assessment of FTO Gene Polymorphism and its Association with Type 2 Diabetes Mellitus in North Indian Populations. *Indian Journal of Clinical Biochemistry*, 1-6.

Nanditha, A., Ma, R. C., Ramachandran, A., Snehalatha, C., Chan, J. C., Chia, K. S., . . . Zimmet, P. Z. (2016). Diabetes in Asia and the Pacific: implications for the global epidemic. *Diabetes care, 39*(3), 472-485.

Nazish, S., Zafar, A., Shahid, R., Albakr, A., Alkhamis, F. A., Aljaafari, D., . . . Al-Mulla, F. A. (2018). Relationship between glycated haemoglobin and carotid atherosclerotic disease among patients with acute ischaemic stroke. *Sultan Qaboos University Medical Journal, 18*(3), e311.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H., & Cho, K. W. (2000). Interaction between Wnt and TGF-β signalling pathways during formation of Spemann's organizer. *Nature, 403*(6771), 781.

Nobrega, M. A. (2013). *TCF7L2* and glucose metabolism: time to look beyond the pancreas. *Diabetes, 62*(3), 706-708.

Nolan, J., & Færch, K. (2012). Estimating insulin sensitivity and beta cell function: perspectives from the modern pandemics of obesity and type 2 diabetes. *Diabetologia, 55*(11), 2863-2867.

Norton, L., Fourcaudot, M., Abdul-Ghani, M., Winnier, D., Mehta, F., Jenkinson, C., & Defronzo, R. (2011). Chromatin occupancy of transcription factor 7-like 2 (*TCF7L2*) and its role in hepatic glucose metabolism. *Diabetologia, 54*(12), 3132-3142.

Oh, K.-J., Han, H.-S., Kim, M.-J., & Koo, S.-H. (2013). CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. *BMB reports, 46*(12), 567.

Ohashi, K., Shibata, R., Murohara, T., & Ouchi, N. (2014). Role of anti-inflammatory adipokines in obesity-related diseases. *Trends in Endocrinology & Metabolism, 25*(7), 348-355.

Osmark, P., Hansson, O., Jonsson, A., Rönn, T., Groop, L., & Renström, E. (2009). Unique splicing pattern of the *TCF7L2* gene in human pancreatic islets. *Diabetologia, 52*(5), 850-854.

Ozougwu, J., Obimba, K., Belonwu, C., & Unakalamba, C. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology, 4*(4), 46-57.

Pang, D., Smith, A., & Humphries, S. (2013). Functional analysis of *TCF7L2* genetic variants associated with type 2 diabetes. *Nutrition, Metabolism and Cardiovascular Diseases, 23*(6), 550-556.

Panunzi, S., Carlsson, L., De Gaetano, A., Peltonen, M., Rice, T., Sjöström, L., . . . Dixon, J. B. (2016). Determinants of diabetes remission and glycemic control after bariatric surgery. *Diabetes care, 39*(1), 166-174.

Park, S., Sadanala, K. C., & Kim, E.-K. (2015). A metabolomic approach to understanding the metabolic link between obesity and diabetes. *Molecules and cells, 38*(7), 587.

Parker, S. C., Stitzel, M. L., Taylor, D. L., Orozco, J. M., Erdos, M. R., Akiyama, J. A., . . . Black, B. L. (2013). Chromatin stretch enhancer states drive cell-specific gene regulation and harbor human disease risk variants. *Proceedings of the National Academy of Sciences, 110*(44), 17921-17926.

Pasquali, L., Gaulton, K. J., Rodríguez-Seguí, S. A., Mularoni, L., Miguel-Escalada, I., Akerman, I., . . . Van De Bunt, M. (2014). Pancreatic islet enhancer clusters enriched in type 2 diabetes risk-associated variants. *Nature genetics, 46*(2), 136.

Pavić, T., Juszczak, A., Pape Medvidović, E., Burrows, C., Šekerija, M., Bennett, A. J., . . . McCarthy, M. I. (2018). Maturity onset diabetes of the young due to HNF1A variants in Croatia. *Biochemia medica: Biochemia medica, 28*(2), 1-11.

Pearson, E. (2009). Translating *TCF7L2*: from gene to function. *Diabetologia, 52*(7), 1227-1230.

Pedroso, J. L., Lucato, L. T., Kok, F., Sallum, J., Barsottini, O. G., & Oliveira, A. S. B. (2015). Association of optic atrophy and type 1 diabetes: clinical hallmarks for the diagnosis of Wolfram syndrome. *Arquivos de Neuro-psiquiatria, 73*(5), 466-468.

Peng, S., Zhu, Y., Lü, B., Xu, F., Li, X., & Lai, M. (2012). *TCF7L2* gene polymorphisms and type 2 diabetes risk: a comprehensive and updated meta-analysis involving 121 174 subjects. *Mutagenesis, 28*(1), 25-37.

Pernicova, I., & Korbonits, M. (2014). Metformin—mode of action and clinical implications for diabetes and cancer. *Nature Reviews Endocrinology, 10*(3), 143.

Phillips, A. (2016). Optimising the person-centred management of type 2 diabetes. *British Journal of Nursing, 25*(10), 535-538.

Pickup, J. C. (2004). Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes care, 27*(3), 813-823.

Prokunina-Olsson, L., Kaplan, L. M., Schadt, E. E., & Collins, F. S. (2009). Alternative splicing of *TCF7L2* gene in omental and subcutaneous adipose tissue and risk of type 2 diabetes. *PLoS One, 4*(9), e7231.

Prokunina-Olsson, L., Welch, C., Hansson, O., Adhikari, N., Scott, L. J., Usher, N., . . . Bonnycastle, L. L. (2009). Tissue-specific alternative splicing of *TCF7L2*. *Human molecular genetics, 18*(20), 3795-3804.

Punthakee, Z., Goldenberg, R., Katz, P., & Committee, D. C. C. P. G. E. (2018). Definition, classification and diagnosis of diabetes, prediabetes and metabolic syndrome. *Canadian journal of diabetes, 42*, S10-S15.

Rao, P., Wang, H., Fang, H., Gao, Q., Zhang, J., Song, M., . . . Wang, W. (2016). Association between IGF2BP2 polymorphisms and type 2 diabetes mellitus: a case–control study and meta-analysis. *International journal of environmental research and public health, 13*(6), 574.

Ren, Q., Han, X., Wang, F., Zhang, X., Han, L., Luo, Y., . . . Ji, L. (2008). Exon sequencing and association analysis of polymorphisms in *TCF7L2* with type 2 diabetes in a Chinese population. *Diabetologia, 51*(7), 1146-1152.

Ren, X., Yin, R., Hou, D., Xue, Y., Zhang, M., Diao, X., . . . Hu, X. (2018). The Glucose-Lowering Effect of Foxtail Millet in Subjects with Impaired Glucose Tolerance: A Self-Controlled Clinical Trial. *Nutrients, 10*(10), 1509.

Riddle, M. C. (2017). Modern sulfonylureas: dangerous or wrongly accused? *Diabetes care, 40*(5), 629-631.

Rizos, C. V., Kei, A., & Elisaf, M. S. (2016). The current role of thiazolidinediones in diabetes management. *Archives of toxicology, 90*(8), 1861-1881.

Ruiz-Narváez, E. A. (2014). Redundant enhancers and causal variants in the *TCF7L2* gene. *European Journal of Human Genetics, 22*(11), 1243.

Sakhneny, L., Rachi, E., Epshtein, A., Guez, H. C., Wald-Altman, S., Lisnyansky, M., . . . Priel, A. (2018). Pancreatic pericytes support β-cell function in a *TCF7L2*-dependent manner. *Diabetes, 67*(3), 437-447.

Sampson, E. M., Haque, Z. K., Ku, M. C., Tevosian, S. G., Albanese, C., Pestell, R. G., . . . Yee, A. S. (2001). Negative regulation of the Wnt–β-catenin pathway by the transcriptional repressor HBP1. *The EMBO journal, 20*(16), 4500-4511.

Sansbury, F., Flanagan, S., Houghton, J., Shen, F. S., Al-Senani, A., Habeb, A., . . . Hattersley, A. (2012). SLC2A2 mutations can cause neonatal diabetes, suggesting GLUT2 may have a role in human insulin secretion. *Diabetologia, 55*(9), 2381-2385.

Saxena, R., Gianniny, L., Burtt, N. P., Lyssenko, V., Giuducci, C., Sjögren, M., . . . Orho-Melander, M. (2006). Common single nucleotide polymorphisms in *TCF7L2* are reproducibly associated with type 2 diabetes and reduce the insulin response to glucose in nondiabetic individuals. *Diabetes, 55*(10), 2890-2895.

Schäfer, S., Tschritter, O., Machicao, F., Thamer, C., Stefan, N., Gallwitz, B., . . . Nijpels, G. (2007). Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (*TCF7L2*) gene polymorphisms. *Diabetologia, 50*(12), 2443-2450.

Schauer, P. R., Bhatt, D. L., Kirwan, J. P., Wolski, K., Aminian, A., Brethauer, S. A., . . . Nissen, S. E. (2017). Bariatric surgery versus intensive medical therapy for diabetes—5-year outcomes. *New England Journal of Medicine, 376*(7), 641-651.

Shahzad, A., Ahmad, M. M., Anwer, I., Ijaz, N., Shahzad, M., & Usman, M. (2018). Gender-specific Knowledge of Diabetes and Its Management Among Patients Visiting Outpatient Clinics in Faisalabad, Pakistan. *Cureus, 10*(8).

Shao, W., Wang, D., Chiang, Y.-T., Ip, W., Zhu, L., Xu, F., . . . Zhang, H. (2013). The Wnt signaling pathway effector *TCF7L2* controls gut and brain proglucagon gene expression and glucose homeostasis. *Diabetes, 62*(3), 789-800.

Sharpe, C., Lawrence, N., & Arias, A. M. (2001). Wnt signalling: a theme with nuclear variations. *Bioessays, 23*(4), 311-318.

Shu, L., Matveyenko, A. V., Kerr-Conte, J., Cho, J.-H., McIntosh, C. H., & Maedler, K. (2015). Decreased *TCF7L2* protein levels in type 2 diabetes mellitus correlate with downregulation of GIP-and GLP-1 receptors and impaired beta-cell function. *Hum Mol Genet, 24*(10), 3004.

Štros, M., Launholt, D., & Grasser, K. D. (2007). The HMG-box: a versatile protein domain occurring in a wide variety of DNA-binding proteins. *Cellular and Molecular Life Sciences, 64*(19-20), 2590.

Sukmawati, D., Tanaka, R., Ito-Hirano, R., Fujimura, S., Hayashi, A., Itoh, S., . . . Daida, H. (2016). The role of Notch signaling in diabetic endothelial progenitor cells dysfunction. *Journal of diabetes and its complications, 30*(1), 12-20.

Tan, A. L., Forbes, J. M., & Cooper, M. E. (2007). *AGE, RAGE, and ROS in diabetic nephropathy.* Paper presented at the Seminars in nephrology.

Thurtell, C., & Mackie, A. (2018). *Rapidly progressive polyuria, polydipsia and headache-an unusual case of central diabetes insipidus.* Paper presented at the Society for Endocrinology Endocrine Update 2018.

Tillil, H., & Köbberling, J. (1987). Age-corrected empirical genetic risk estimates for first-degree relatives of IDDM patients. *Diabetes, 36*(1), 93-99.

Tiwari, A. K., & Rao, J. M. (2002). Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. *Current science*, 30-38.

Tuomilehto, J., Lindström, J., Eriksson, J. G., Valle, T. T., Hämäläinen, H., Ilanne-Parikka, P., . . . Rastas, M. (2001). Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *New England Journal of Medicine, 344*(18), 1343-1350.

Venables, M. C., & Jeukendrup, A. E. (2009). Physical inactivity and obesity: links with insulin resistance and type 2 diabetes mellitus. *Diabetes/metabolism research and reviews, 25*(S1), S18-S23.

Wang, J., Zhang, J., Li, L., Wang, Y., Wang, Q., Zhai, Y., . . . Hu, D. (2013). Association of *rs12255372* in the *TCF7L2* gene with type 2 diabetes mellitus: a meta-analysis. *Brazilian Journal of Medical and Biological Research, 46*(4), 382-393.

Wang, Y., Li, G., Goode, J., Paz, J. C., Ouyang, K., Screaton, R., . . . Montminy, M. (2012). Inositol-1, 4, 5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes. *Nature, 485*(7396), 128.

Weedon, M. (2007). The importance of *TCF7L2*. *Diabetic Medicine, 24*(10), 1062-1066.

Wegner, L., Hussain, M. S., Pilgaard, K., Hansen, T., Pedersen, O., Vaag, A., & Poulsen, P. (2008). Impact of *TCF7L2* rs7903146 on insulin secretion and action in young and elderly Danish twins. *The Journal of Clinical Endocrinology & Metabolism, 93*(10), 4013-4019.

Weise, A., Bruser, K., Elfert, S., Wallmen, B., Wittel, Y., Wöhrle, S., & Hecht, A. (2009). Alternative splicing of *TCF7L2* transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/β-catenin targets. *Nucleic acids research, 38*(6), 1964-1981.

Welters, H. J., & Kulkarni, R. N. (2008). Wnt signaling: relevance to β-cell biology and diabetes. *Trends in Endocrinology & Metabolism, 19*(10), 349-355.

Wirström, T. (2012). *Nutritional and dysmetabolic factors with potential impact on type 2 diabetes: epidemiological and molecular studies*: Inst för molekylär medicin och kirurgi/Dept of Molecular Medicine and Surgery.

Wu, J. H., Foote, C., Blomster, J., Toyama, T., Perkovic, V., Sundström, J., & Neal, B. (2016). Effects of sodium-glucose cotransporter-2 inhibitors on cardiovascular events, death, and major safety outcomes in adults with type 2 diabetes: a systematic review and meta-analysis. *The lancet Diabetes & endocrinology, 4*(5), 411-419.

Yang, X., Coulombe-Huntington, J., Kang, S., Sheynkman, G. M., Hao, T., Richardson, A., . . . Murray, R. R. (2016). Widespread expansion of protein interaction capabilities by alternative splicing. *Cell, 164*(4), 805-817.

Yao, H., Wang, Z., Wang, T., Ma, Y., Su, Y., Ma, Q., . . . Zhu, J. (2015). Association of *TCF7L2* genetic polymorphisms with type 2 diabetes mellitus in the Uygur population of China. *International journal of environmental research and public health, 12*(9), 11797-11814.

Yu, O., Azoulay, L., Yin, H., Filion, K. B., & Suissa, S. (2018). Sulfonylureas as initial treatment for type 2 diabetes and the risk of severe hypoglycemia. *The American journal of medicine, 131*(3), 317. e311-317. e322.

Zhang, X., Yang, S., Chen, J., & Su, Z. (2018). Unraveling the regulation of hepatic gluconeogenesis. *Frontiers in endocrinology, 9*.

Zheng, Y., Ley, S. H., & Hu, F. B. (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology, 14*(2), 88.

Zhou, Y., Park, S.-Y., Su, J., Bailey, K., Ottosson-Laakso, E., Shcherbina, L., . . . Fadista, J. (2014). *TCF7L2* is a master regulator of insulin production and processing. *Human molecular genetics, 23*(24), 6419-6431.

Zimmet, P., Alberti, K. G., Magliano, D. J., & Bennett, P. H. (2016). Diabetes mellitus statistics on prevalence and mortality: facts and fallacies. *Nature Reviews Endocrinology, 12*(10), 616.

Zimmet, P. Z., & Alberti, K. G. M. (2016). Epidemiology of diabetes—status of a pandemic and issues around metabolic surgery. *Diabetes care, 39*(6), 878-883.