GENETIC ANALYSIS OF ANGIOTENSIN CONVERTING ENZYME II GENE (ACE II) ASSOCIATION POLYMORPHISM IN TYPE 2 DIABETES MELLITUS

BY

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MS HEALTHCARE BIOTECHNOLOGY

SESSION 2015-2017

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A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Healthcare Biotechnology

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In the Name of Allah, the Most Gracious, the Most Merciful

O Allah! The Lord of the people, the Remover oftrouble! (Please) cure (Heal) (this patient), for You are the Healer. None brings about healing but You; a healing that will leave behind no ailment.

- Bukhari (Book #71, Hadith #638)

Dedicated to my beloved parents for their unconditional love, support and partnership for my success and to all those who keep trying putting me down!

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Thanks a lot you all!

Zainab Mir

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LIST OF ACRONYMS

ACE	Angiotensin Converting Enzyme
AT_1	Angiotensin Type 1
AT_2	Angiotensin Type 2
BSR	Blood Sugar Random
cDNA	Complimentary DNA
COOH	Carboxylic Acid
DD	Deletion-Deletion
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DPP	*Dipeptidyl peptidase*
EDTA	Ethylenediamenetetraacetic acid
GLP	Glucagon-like Peptide
GLUT	Glucose Transporter
GTT	Glucose Tolerance Test
IAPP	Insulin Associated Amyloid Polypeptide
II	Insertion-Insertion
IRS	insulin receptor substrate
I/D	Insertion-Deletion
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction

RAS	Rennin-Angiotensin System
SDS	Sodium Dodecyl Sulphate
SGLT2	sodium/glucose cotransporter 2
T2DM	Type 2 Diabetes Mellitus

ABSTRACT

Type 2 Diabetes Mellitus is a metabolic dysfunction that target many organs thus creating complications and linking to increased cardiovascular diseases. This disorder is progressing day by day worldwide. Currently, many studies have supported the involvement of Angiotensin Converting Enzyme II Gene (ACE II) in diabetes, which is a major compound in Rennin-Angiotensin System (RAS) involved in the activation of majority pathophysiological impacts of RAS. This study was designed to screen and study the ACE II gene in the blood samples type 2 diabetic patients and normal healthy individuals in order to investigate the specific genotypes in the diseased and healthy individuals by observing association polymorphism of ACE II gene as three different polymorphs; Insertion (II), insertion-deletion (I/D) and deletion (DD). The results showed more involvement of D allele in both cases in which more DD genotype was analyzed to be present in healthy individuals whereas type 2 diabetic patients possessed I/D genotype more than the other two. This data analysis could lead to the formation of ACE II inhibitors to avoid the progression of Type 2 Diabetes Mellitus.

Chapter1

Introduction

1.1 Type 2 Diabetes Mellitus (T2DM)

Diabetes Mellitus is a persistent metabolic dysfunction which results into hyperglycemia, targeting many organs thus creating complications and linking to increased cardiovascular morbidity and mortality. In type 2 diabetes, the non insulin dependent diabetes is a chronic disorder that affects the metabolic pathway in some way and the resisting effect of insulin which is a restrictive hormone that regulates the glucose movement into the cells or the insulin is not produced enough to keep glucose level normal(Mayer-Davis et al., 2017).

The insulin is not properly consumed by the Type 2 Diabetic patients. This type could be developed at any age, mainly diagnosed in older and middle-aged people. It is the most common type of diabetes worldwide (Dall et al., 2009). The precursor of diabetes mellitus type 2 is usually insulin resistance. Type 2 diabetic patients do not have much insulin produced to function properly because of which the body cells do not respond to insulin well. Pancreas are therefore stimulated for more insulin production but are unable to stop blood glucose levels from rising high (Sreedevi & Kumar, 2017).

Approximately 90% diabetes cases around the globe are type 2. Some diabetic patients may reduce their type 2 symptoms by weight loss, which is following of a healthy diet and performing exercises along with the monitoring of their blood sugar levels (Feinglos &

Bethel, 2008). However, type 2 diabetes mellitus is an ongoing disease which gets worse gradually if not taken care of and the patient ends up taking insulin daily. Obese and overweight people are at a more risk of developing this type as compared to the people with a healthy body weight. An overweight body releases chemicals which destabilizes the metabolic and cardiovascular systems (Ganguly et al, 2015). Individuals with abdominal obesity and central obesity are at more risk of acquiring type 2 diabetes mellitus. Also being physically inactive and consumption of fatty or junk food and soda contribute to this disease development. Other than obesity, men with low testosterone level are also at a risk of acquiring type 2 diabetes mellitus as a research has shown to have links between low testosterone levels and insulin resistance (Grossmann et al., 2008).

T2DM symptoms include persistent urination, also called polyuria, increased hunger (polyphagia), along with increased thirst; polydipsia and also itchiness, peripheral neuropathy, blurred vision, fatigue and recurrent vaginal infections (Masharani & German, 2004). However, many patients show zero symptoms in the first few years and are diagnosed upon a routine checkup. A diabetic patient may develop a condition in which the blood glucose level is very high and is linked to a lessened level of consciousness along with low blood pressure.

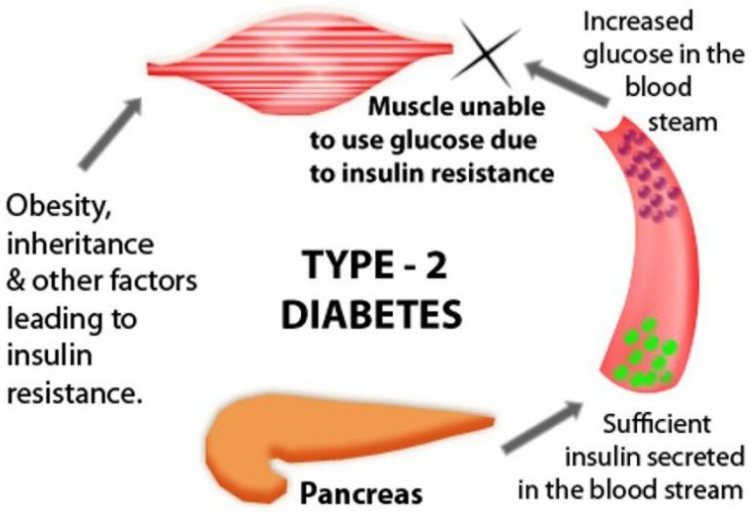


Figure 3.1 : Function of Pancreas in Type 2 Diabetes

The life expectancy of a diabetic patient becomes ten year shorter because of the number of associated complications mainly that of the heart and also a 20 fold increase of amputations of the lower limb (Melmed et al, 2011). T2DM is also the largest element of kidney failure and blindness and has also been observed to be associated with dementia and cognitive dysfunction such as Alzheimer's disease (Pasquier, 2010; Ripsin et al, 2009).

1.2 Epidemiology of Type 2 Diabetes Mellitus

In the last two decades, it was thought that T2DM is a metabolic disorder and has become more common in obese individuals (Copeland et al., 2013).The World Health Organization estimated the diabetic population to be 9% whereas 90% of the calculated percentage was that of type 2 diabetes mellitus (Whiting et al, 2011). 5 million deaths per year are caused due to T2DM. Hence, it is expected that T2DM will be the 7th cause of death by the year 2030 (D'Adamo & Caprio, 2011). With an increased prevalence of the pre-diabetic

conditions the increased predominance of type 2 diabetes mellitus in the obese children is paralleled. 21% adults and 25% of young obese children were observed to have T2DM in a population. In a large sample of overweight children and adults, glucose metabolism alterations were observed to be 12.4% (Flint & Arslanian, 2011).

The prevalence of T2DM is increasing rapidly in our country Pakistan as well. The disease progression not only increases the economic burden of the patient but also the country they reside in. According to the recent studies, the current predominance of type 2 diabetes mellitus in Pakistan is 11.78% (Morris et al., 2012). The females have a prevalence of 9.19% and that of the males is 11.20%. In Sindh, type 2 diabetes mellitus prevalence in males is 16.2% and 11.70% I females thus having a higher percentage of type 2 diabetic patients than other provinces (Meo et al, 2015).

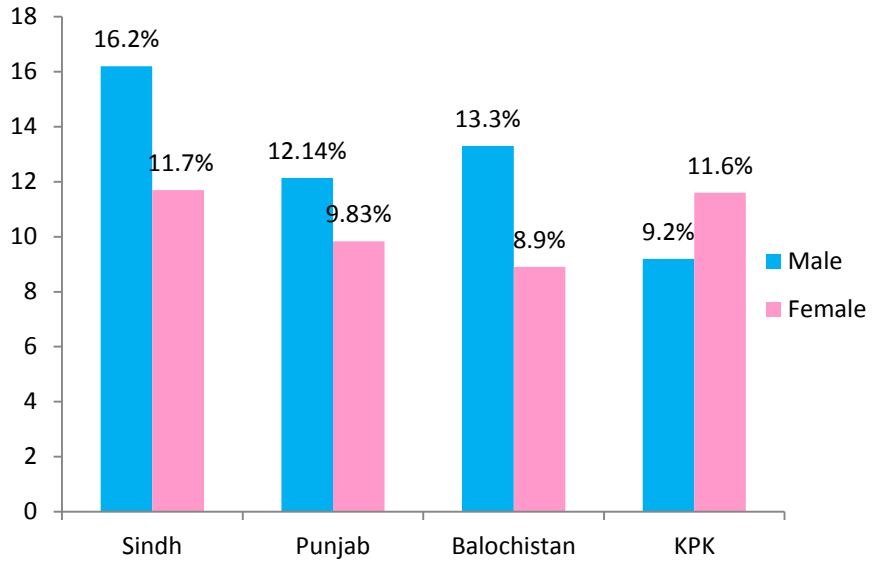


Figure 1.4: T2DM Prevalence in Pakistan

In the civic areas of Pakistan, the overall predominance of type 2 diabetes mellitus is 14.81% in males and 10.3% in females. Pakistan should include preventive measures for diabetes in National Health Policy in order to lessen the prevalence of the disease (Khan & King, 1999).

1.3 Pathophysiology of Type 2 Diabetes Mellitus

T2DM results due to the combination of reduced secretions and increased requirements of insulin. However, the relative contribution is individual specific (Scheen & Lefèbvre, 1996). With an increasing age, the insulin secretion declines which depicts the act of the associated genes with diabetes, majority of these affect beta cell function instead of tissue delicacy to insulin (Defronzo, 1997). Accumulation of IAPP (islet-associated amyloid peptide) in and around the islets is another factor that leads to diabetes along with insulin resistance. The overproduction of glucose by the liver and less uptake of glucose in the peripheral tissues causes hyperglycemia (Kahn, 2003). In the early stages of diabetes, secretion of insulin is increased but it declines with time due to progressive beta cell failure. Any abnormality that occurs lipid metabolism is another factor which leads to T2DM (Gerich, 1998).

1.3.1 Insulin Secretion and Resistance

Beta cell regeneration decreases in adults thus showing a visible decrease in beta cell mass with developing age along with an increased risk of T2DM (DeFronzo, 1988). Diabetes associated genes have an important role in maintaining the beta cell function. In the early stages of T2DM, the increased insulin secretion is rest behind the amount required to assign high glucose levels. Therefore, increased glucose drive

is important to keep the insulin secretion normal. Synchronized insulin release is lost at the early stage of diabetes mellitus development and is one of the factors that lead to glucose toxicity (Greenberg & McDaniel, 2002).

1.3.2 Insulin Associated Amyloid Polypeptide (IAPP)

The material accumulating around the islets in the diabetic patients are shown to be associated with insoluble polymers of IAPP, which are harmful and affect the beta cell function, thus leading to the cause (Scheen & Lefebvre, 1992).

1.3.3 Hormonal Antagonism

Insulin has a partner hormone called glucagon which helps it in the regulation of the hepatic secretion of glucose (Polonsky, 1995). Increased glucagon has a vital role in the T2DM. It is involved in the tissue injury and is also majorly responsible for the increased resistance of insulin and also physical stress associated hyperglycemia (Polonsky, 1995; Scheen & Lefebvre, 1992).

1.3.4 Cytokine Release

Tissue inflammation is found to be a consequence of obesity and also causes the inflammatory cytokines to release which includes tumor necrosis factor – α along with resistin which diminishes the effect of insulin (Scheen & Lefebvre, 1992).

1.3.5 Body Fat Distribution

Insulin resistant individuals who are obese as well are prone to triglyceride

accumulation, the extent of which correlates with insulin resistance (Polonsky, 1995). Insulin signaling is interfered by the fats present intracellularly which contributes to the resistance of insulin and is a powerful predictor of diabetes risk (Greenberg & McDaniel, 2002).

1.4 Clinical Diagnosis of T2DM

It is important for diabetes mellitus to be analyzed early so that treatment can be started as soon as possible to treat it well. A few tests are done for the diagnosis of diabetes (Association, 2016).

1.4.1 Glycated Haemoglobin (HbA1c)

Patients who have been diagnoses with diabetes mellitus are tested for their HbA1c levels to check how properly their glucose levels are being controlled. The HbA1c test tells about the average glucose levels carried on for past two to three months. The diabetic patients should get their HBA1c measured twice a year but more frequently if the patient has been recently analyzed with diabetes mellitus, glucose level remains too high or the treatment plan has been altered. This test could be carried out at any time irrespective of any special preparations, like fasting. However, this test is not applicable in case of pregnancy.

An HbA1c level of 6.5% or higher indicates T2DM. Whereas, a person showing HbA1c level between 6% to 6.4% would likely be at a higher risk of developing

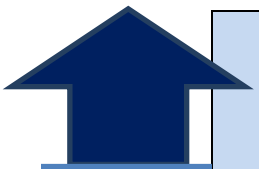
diabetes mellitus and may also be called as pre-diabetic (Selvin et al., 2010).

1.4.2 Random Blood Sugar Test

In this test a sample of blood is taken randomly, irrespective of the time, to measure the glucose level in blood. The values are expressed in milligrams per deciliter (mg/dL) or millimoles per liter (mmol/l). A random sugar level of 200md/dl (11.1mmol/l) or above that will depict diabetes coupled with the symptoms of extreme thirst and frequent urination (Landon et al., 2009; Selvin et al., 2010).

1.4.3 Fasting Blood Sugar Test

This test records the sugar levels of patients in a fasting condition. A patient is required to not consume any food or drink for 8-12 hours and may also be asked to ignore certain medications before the test is performed. A fasting sugar level of 100-125 mg/dL (5.6 – 6.9 mmol/l) is a pre-diabetic condition which shows that a patient is at a high risk of developing diabetes. Whereas, the values recorded 126md/dL (7 mmol/l) or above indicate diabetes mellitus (Landon et al., 2009).



	A1C (percent)	Fasting Plasma Glucose (mg/dL)	Oral Glucose Tolerance Test (mg/dL)
Diabetes	6.5 or above	126 or above	200 or above
Pre-diabetes	5.7 to 6.4	100 to 125	140 to 99
Normal		99 or below	139 or below

Table 1.1: Blood Test Levels for Diagnosis of DiabetesMellitus: Within the prediabetes range, the higher the test result the greater the risk of diabetes

1.4.4 Glucose Tolerance Test (GTT)

A glucose tolerance test which is also termed as oral glucose tolerance test (OGTT), A blood sample is taken and the blood glucose level is monitored after which a sweet glucose drink is consumed by the patient. The blood glucose is again measured after two hours post drinking the sweet drink to record the glucose level (Landon et al., 2009; Nichols, Bell, Kimes, & O'Keeffe-Rosetti, 2016).

The test will show if a person has glucose tolerance or diabetes depending upon the glucose level in the patient's blood before and after consuming the glucose drink. Glucose level is measured in millimoles per litre (mmol/l). A non-diabetic patient should have a glucose level less than 6mmol/l before the test and less than 7.8 mmol/l two hours post testing. Whereas, a diabetic patient will have more than 7mmol/l before the test and 11mmol/l post two hours of the test. Medication may be recommended to lower the blood glucose levels and help keep it under control (Nichols et al., 2016).

1.5 Risk Factors involved in Type 2 Diabetes Mellitus

Certain factors are involved in inducing T2DM which can be controlled to prevent the development of the disease.

1.5.1 Weight

Obesity is a primary cause of type 2 diabetes mellitus. The cell resistance to insulin increases as the development of fatty tissue increases. However, being overweight does not always lead to T2DM (Gress et al, 2000).

1.5.2 Fat Distribution

If the body stocks fat in abdomen, the chances of developing T2DM is higher than the fat storage anywhere else such as thighs or hips (Espeland, 2007).

1.5.3 Inactivity

Physical activity controls the body weight by utilizing glucose as a source of energy and make cells more sensitive to insulin. The more active a person is, less are the chances for developing T2DM and vice versa.

1.5.4 Family History

If a person has a family history of T2DM, he has more chances to acquire T2DM.

1.5.5 Age

The risk of T2DM increases with age, especially after 45 as people do not exercise much and gain weight thus losing muscle mass. Other than this T2DM is progressing among children and younger adults as well (Stratton et al., 2000).

1.5.6 Gestational Diabetes

If a pregnant woman develop diabetes mellitus, chances are that it will progress to T2DM post-delivery and if the baby weighs more than 9 pounds the risk of developing type 2 diabetes mellitus gets higher.

1.5.7 Polycystic Ovarian Syndrome

Women having polycystic ovarian syndrome (PCOS), conditions like excess hair growth, irregular menstrual cycle and obesity leads to the risk of diabetes (Inzucchi et al, 2014).

1.6 Treatment of Type 2 Diabetes Mellitus

T2DM can be treated by adopting a lifestyle that supports healthy consumption of food and burning calories by exercising on daily basis. Blood sugar monitoring should be regular and medication for diabetes mellitus should be properly followed.

1.6.1 Healthy Eating

Low fat and high fiber diet should be consumed such as fruits, vegetables, whole grains and refined carbs with a fewer animal products (Control & Trial, 2005). Low glycemic index foods also help in stabling the blood sugar level as they are high fiber products. A healthy meal plan can be taken according to the body type according to which proper intake of carbohydrates should be done (Rother, 2007).

1.6.2 Physical Activity

Regular exercises should be performed by following a proper program to regulate various constituent levels in the body. Walking, swimming and biking can aid well in making a physical activity routine for diabetic patients (Rother, 2007). Aerobic and strength training exercises for at least 30 mins can help regulating body functions by lowering blood sugar level.

1.6.3 Blood Sugar Monitoring

Sugar level should be monitored on daily basis to keep a record of the patient's condition so that the glucose level remains in the target range (Redekop et al.,

2002).

1.6.4 Bariatric Surgery

If the body mass index of a person is greater than 35,baritric surgery which is a weight loss surgery may be performed on that individual. Blood sugar levels get normal depending upon the type of surgery performed. The drawback of this treatment option is the risk of death. Drastic life changes are required post-surgery which an individual may not follow and may lead to long term complications (Bibeau & Nelson, 2016).

1.6.5 Medication

Patients of type 2 diabetes mellitus may also achieve the target range of blood sugar through certain medication prescribed by the doctor depending upon an individual's condition. The type of medicine depends upon the symptoms and certain factors which include the glucose level and other health problems (Capoccia et al, 2016; Mann et al, 2009).

There are classes of drugs which are prescribed according to a person's condition. These include:

- Metformin (Glucophage, Glumetza) – the first medication prescribed which makes the body tissue sensitivity to insulin better and more effective and also lowers glucose production

- Sulfonylureas (Glynase, DiaBeta) – helps in more insulin secretion
- Meglitinides (Starlix, Prandin) – stimulate pancreas to secrete more insulin. These are fast acting but effect the body for a shorter time.
- Thiazolidinediones (Avandia, Actos) – make body tissues sensitive to insulin. This class is linked to weight gain and is not the first choice treatment.
- DPP-4 inhibitors (Januvia, Stiagliptin) – reduce blood sugar levels
- GLP-1 receptor agonists (Byetta, Victoza) – slow digestion to lower the glucose levels in the blood
- SGLT2 inhibitors (Farziga) – prevent kidneys from reabsorption of glucose into the blood
- Insulin Therapy – A few of the type 2 diabetic patients require insulin and take one long acting shot once a day mostly at night.

No treatment can cure diabetes mellitus so patients consuming insulin for diabetes and other medications should not stop unless advised by the physician.

1.7 **Complications associated to Type 2 Diabetes Mellitus**

T2DM can be ignored in the early stages when a person feels totally fie but it may affect major organs including heart, blood vessels, eyes, nerves and kidneys. Controlling the glucose level can prevent acquiring these complications. Long term complications develop with time which may be disabling or even life threatening.

1.7.1 Heart and Blood Vessel Disease

The chances of developing various heart-related problems increase as the diabetes progress which also increases heart dysfunctions with angina, heart attack, high blood pressure and narrowing of arteries (Zinman et al., 2015).

1.7.2 Nerve Damage (Neuropathy)

Increased levels of glucose can rupture walls of blood capillaries which provide blood to nerves in the legs which then causes burning or aches starting from the toes or fingers tips and gradually moves upwards. Poorly controlled blood glucose can cause the person to drop sense of feeling in the arms and legs that are affected (Sjöström et al., 2014). Nerve damage of the nerves involved in digestion can cause vomiting, nausea, diarrhea or constipation whereas erectile dysfunction can be caused to men.

1.7.3 Kidney Damage (Nephropathy)

Kidneys have millions of blood capillaries that excrete waste from blood which is damaged because of the development of DM. Too muchdestruction can lead to kidney failure or last stage kidney problem which then requires a transplant or dialysis(Marso et al., 2016).

1.7.4 Eye Damage

Diabetes can also affect the eye blood vessels causing diabetic retinopathy thus causing blindness and other serious vision abnormalities such as glaucoma and

cataracts.

1.7.5 Foot Damage

Lessened blood flow to the feet increase risks of various lower limb complications. Untreated blisters and cuts eventually lead to bad infections which may take lots of time to heal. Severe damage leads to toe, foot or leg amputations (Green et al., 2015).

1.7.6 Skin and Hearing Conditions

Diabetes mellitus makes the patient more prone to skin problems including fungal and bacterial infections. Hearing issues become common in people with T2DM.

1.7.7 Alzheimer's Disease

Type 2 diabetes mellitus increase the chances of developing Alzheimer's disease (Zoungas et al., 2014). The risks are greater when the control of glucose in the body is poor. Alzheimer's and T2DM share various genetic links.

1.8 Rennin-Angiotensin System (RAS)

Rennin Angiotensin System (RAS) has two main types; local (non-circulatory; tissue specific) and systemic (circulatory) (Dzau, 1988). The circulatory RAS is associated in blood

pressure regulation in which the rennin is produced and secreted via special cells of the kidney into the blood stream. This makes the rennin convert the inactive Angiotensinogen to an active form known as Angiotensin-I (Adachi, Numakawa, Richards, Nakajima, & Kunugi, 2014). An enzyme called Angiotensin Converting Enzyme (ACE) converts Angiotensin-I to Angiotensin-II thus promoting the blood regulation. Angiotensin-II releases Aldosterone acts as a vasoconstrictor which makes the kidneys to retain salt and water resulting in hypertension. This activity is carried out by two receptors that are G-protein coupled; the Angiotensin type 1 (AT_1) receptor which is linked to the function of Angiotensin-II. Whereas the Angiotensin type 2 (AT_2) receptor is involved in the aldosterone secretion from the adrenal cortex (Mogensen et al., 2000). Therefore, RAS has an essential role in the balancing of sodium, renal and systemic vascular resistance and extracellular fluid volume thus being the potent arterial blood pressure regulator.

This involvement of RAS in blood pressure regulation helps in the curing of patients having hypertension, left ventricular dysfunction, heart failure, liver cirrhosis, pulmonary and systemic edema scleroderma, migraines and diabetic neuropathy by inhibiting the RAS pathway (Y. C. Li et al., 2002).

1.9 RAS and Diabetes

The relationship of the RAS with the endocrine system is observed by the working of Angiotensin-II in diabetes and metabolic disorder. The continuous linking of diabetes mellitus (DM) with hypertension, nephropathy, retinopathy, and cardiovascular disease has embroiled the RAS in the starting and development of these disorders (Ribeiro-Oliveira Jr et

al., 2008). This has been proved by clinical testing in which RAS inhibitors fundamentally diminished the frequency of vascular problems in diabetic patients. These upgrades seem to come about because of defensive activities upon skeletal muscle and pancreatic islets, and furthermore from improved insulin affectability related with diminished adipocyte size, and increased transcapillary glucose transport. Ang II can likewise make to dvelop insulin resistance by meddling with the insulin-empowered increment in insulin receptor substrate 1-related PI3K activity (Bangalore, Fakheri, Toklu, & Messerli, 2016).

Additionally, the renal RAS is clearly activated in diabetes mellitus, with progressive tissue Ang II that induces the progression of diabetic nephropathy, which is a major reason for end-stage renal disorder. RAS blockage could subsequently diminish tissue Ang II levels, with advantageous impacts on heart and kidney function (Schievink et al., 2016).

The metabolic disorder includes obesity, dyslipidemia, insulin resistance and hypertension, regularly with hypertriglyceridemia and diminished high-density lipoprotein, expanded C-reactive protein and also hyperuricemia(Qian et al., 2018). These highlights are frequently connected with endothelial cell abnormality with hindered control of increased adhesiveness of leukocytes, vascular tone,, and rapid development of growth factors and cytokines. These procedures have been involved in the advancement of diabetes, heart disappointment, hypertension, atherosclerosis, and kidney failure. In this failure, RAS components, for the most part Ang II, have a major part in endothelial cell dysfunction, irritation, proliferative impacts and insulin resistance (Márquez, Riera, Pascual, & Soler, 2014).

1.10 Angiotensin Converting Enzyme Gene (ACE)

Angiotensin-I Converting Enzyme gene is present on chromosome 17q23 and is majorly studied because of its prominent role in Rennin-Angiotensin System (RAS) of its conversion from Angiotensin-I to Angiotensin-II thus activating the Aldosterone (Yusuf et al., 2000).

The ACE gene is of 21kb which consists of 26 exons and 25 introns (Raimondi et al., 2016). In case of insertion/deletion (1D) polymorphism of an Alu repeat sequence of 312bp, three possible genotypes appear which consists of a homozygous variant 'DD', ancestral 'II' and heterozygous 'ID'. The 'DD' genotype is an independent risk factor in various cardiovascular disorders such as Myocardial Infarction, Ventricular Hypertrophy and Hypertrophic Cardiomyopathy along with chronic renal diseases, Congenital Urological Anomalies and Diabetic Neuropathies (Moradzadegan, Vaisi-Raygani, Nikzamir, & Rahimi, 2015). The ancestral genotype 'II' leads to a lower definition of Angiotensin Converting Enzyme (ACE) and the ACE variant genotype 'DD' promote a higher definition of Angiotensin Converting Enzyme (ACE) (Foo, Coppack, Denver, Bulmer, & Yudkin, 2015).

Two isoforms which are the splice variants of ACE gene are present in humans; one is the somatic ACE, found in the tissue possessing local RAS and circulation whereas the second one is called Germinal or Testicular ACE found in sperms (Shang et al., 2016). The main difference between both variants is that of their structures which depends on the basis of their catalytic domains possessed by the genes. Germinal ACE consists of one whereas the somatic ACE has two catalytic domains. The difference seems to have been arisen by the

gene duplication event which might have occurred during evolution (Malueka et al., 2017).

1.11 ALU Repeats

Alu insertions are ~300 bp long repetitive sequences, classifies as short interspersed elements (SINEs). The total human genome is made up of 5-10% of these repeats which are derived ancestrally from 7SL RNA gene, present in the form of heterodimer of two subunits which spread into new chromosomal locations via retropositioning(Bakshi, Herke, Batzer, & Kim, 2016). The Alu polymorphic insertions at specific locations occur as unique events thus potentiating their role as stable genetic markers. No mechanism has been found for their removal therefore the change becomes permanent. However, in rare cases of deletions, the changes are detectable as the two cut points for deletion would be present exactly at the points of insertion (Jordà et al., 2017).

Alu repeats have also been studied in disease occurrence as their insertion in coding regions disrupts gene structure hence contributing to pathogenesis (Lubelsky & Ulitsky, 2017). Alu repeats are also found in the non-coding DNA regions thus being involved in regulatory functions as in the case of ACE gene (Payer et al., 2017).

1.12 Impact of the Study

To highlight the importance of Type 2 Diabetes Mellitus progressing in the population of Pakistan, in order to find a better and economical option for its treatment among a large number of population

1.13 Objective of Study

This study is aimed to examine the association of Angiotensin Converting Enzyme II for its insertion and deletion genetic polymorphism in Type 2 Diabetes Mellitus patients in Pakistan.

Chapter 2

Review of Literature

2.1 Insulin Actions

Insulin is an anabolic peptide hormone emitted by the b-cells of the pancreas that assumes a basic part in the regulation of human digestion. Despite the fact that insulin is generally seen as a glucose homeostasis managing hormone, it is presently known to have a considerably more extensive pleiotropic part (Wilmot et al., 2017). An insulin-like signaling system exists in all metazoans, and controls developmentally saved procedures including proliferation and life expectancy (Ji et al., 2015).

The idea that insulin demonstrations by advancing glucose transport over the layer of target cells (as opposed to acting straight forwardly on chemicals of go-between digestion of glucose) was built up in 1949 by the famous trial of Rachmiel Levine and partners, who demonstrated that insulin uniquely expanded the volume of dispersion of non-metabolisable galactose in gutted nephrectomized dogs from 45-47% of body weight to 75%, a figure near that of aggregate body water (De Meyts, 2016). From this discovering they proposed the accompanying working theory: "Insulin follows up on the cell membrane of specific tissues (skeletal muscle, and so forth.) in such a way, to the point that the exchange of hexoses (and maybe different substances) from the extracellular liquid into the cell membrane is encouraged (De Meyts, 2016; García-Cáceres et al., 2016). The intracellular destiny of the hexoses relies on the accessibility of metabolic systems for their change. On account of

glucose, dissimilation, glycogen stockpiling, and change to fat are optionally empowered by the velocity of its entrance into the cell".

This major calculated progress made ready to the idea that insulin follows up on a particular cell layer receptor. The receptor was first described by radio ligand restricting examinations in the mid 70's, and by point by point biochemical investigations in the mid 80's that set up the glycoprotein nature and subunit structure of the receptor (Samuel & Shulman, 2016). Ensuing the exhibit in 1982 by Ora Rosen's gathering that a tyrosine kinase and insulin receptor were nearly connected, a few gatherings demonstrated that the insulin receptor is a tyrosine kinaseitself, a chemical that catalyzes the exchange of the ATP's G phosphate to tyrosine buildups on protein substrates, the first being simply the receptor. In 1985, the cloning of the insulin receptor cDNA by the gatherings of Bill Rutter and Axel Ullrich set up that the insulin receptor surely has a place with the superfamily of receptor tyrosine kinases (RTKs) (Cartee, 2015).

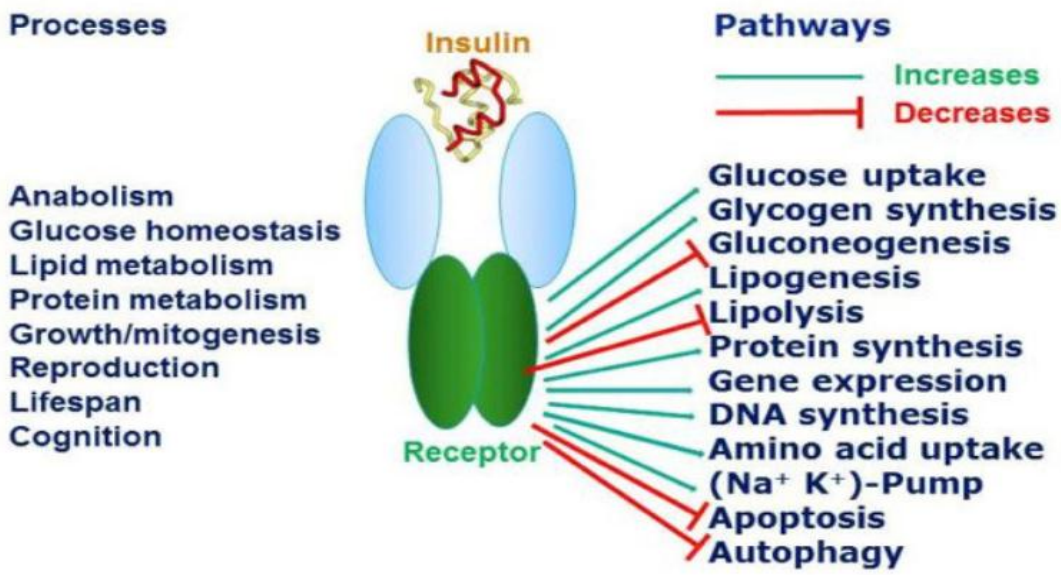


Figure 2.1: Insulin Actions through the Insulin Receptor

2.2 Transduction Pathway

The working of a signal transduction pathway depends on extra cellular signaling that makes a reaction which is the cause of other consequent reactions, subsequently making a chain response, or course (Gomes & Blenis, 2015). Over the span of signaling, the cell utilizes every reaction for achieving some sort of a reason en route. Insulin signal transduction is a typical case of signal transduction pathway mechanism (Kolch, Halasz, Granovskaya, & Kholodenko, 2015).

Insulin is delivered by the pancreas in Islets of Langerhans, in which there are beta-cells, which are in charge of creation and capacity of insulin. Insulin is emitted as a reaction instrument for checking the expanding overabundance measures of glucose in the blood (White & Yenush, 1998).

Glucose increases after sustenance utilization. This is essentially because of the intake of carbohydrate, yet to a substantially lesser degree protein consumption (Avruch, 1998). Depending upon the tissue write, the glucose make its way to the cell through encouraged or uninvolved dispersion (White & Yenush, 1998). In fat and muscle tissue, glucose enters through GLUT 4 receptors by means of encouraged dissemination. In cerebrum, retinaandkidney, glucose enters inactively. In the beta-cells of the pancreas, glucose make its way through the GLUT 2 receptors (Cheatham & Kahn, 1995).

2.3 Molecular Mechanism of Insulin Resistance

Insulin resistance infers that the body's cells (basically muscle) fails the affectability to insulin. At the atomic level, a membrane detects insulin via insulin receptors, with the signal engendering through a course of particles all in all known as PI3K/Akt/mTOR signaling pathway. Recent examinations proposed that the route may work as a bistable switch under physiologic circumstances for specific types of cells, and insulin reaction may well be a limit phenomenon. The pathway's sensitivity to insulin might be blunted by numerous components, for example, free fatty acids, causing insulin resistance. From a more extensive point of view, be that as it may, affectability tuning (counting affectability lessening) is a typical practice for a life form to adjust to the changing condition or metabolic conditions (Laakso, Edelman, Brechtel, & Baron, 1990). Pregnancy, for instance, is an unmistakable difference in metabolic circumstances, because of which the mother needs to diminish her insulin sensibility of muscles to save more glucose for the brains (mother and fetus’s mind). This can be accomplished via raising the reaction limit (i.e., putting off the beginning of affectability) by emitting placental development factor to

meddle with the communication between PI3K and insulin receptor substrate (IRS), which is the embodiment of the supposed customizable edge theory of insulin resistance (Hanson & Pratley, 2000).

2.4 Insulin Resistance and Rennin-Angiotensin System

Insulin resistance upregulates the renin-angiotensin system (RAS), which adds to the pathogenesis of hypertension, heart diseases, and atherosclerosis. RAS restraint diminishes cardiovascular and renal morbidity and mortality and the rate of new-beginning type 2 diabetes (Mcfarlane, Banerji, & Sowers, 2001). To a similar degree, angiotensin II debilitates insulin flagging, instigates aggravation through the atomic factor-kappaB pathway, and decreases nitric oxide accessibility and encourages vasoconstriction, prompting insulin resistance and endothelial dysfunction (Tamemoto et al., 1994). In this manner, the RAS, insulin resistance, and inflammation propagate each other and coordinately add to endothelial dysfunction, vascular damage, and atherosclerosis.

2.5 Angiotensin-II Converting Enzyme Gene in Rennin-Angiotensin System (RAS)

The implication of the renin-angiotensin system (RAS), in the direction of the cardiovascular framework is outstanding for a long time. Numerous pharmaceuticals have been produced to treat a few pathologies accordingly, e.g. diabetes related hypertension, hypertension and heart failure and RAS is developing as one of the real focus in the treatment of cardiovascular diseases. In this manner, the entire dynamic of RAS must be assessed (Kanakamedala, 2007). A better comprehension of the components of RAS will

open new skylines for novel medication treatment by RAS inhibition in treating type 2 diabetes around the world. The significance of the Renin-Angiotensin system (RAS) and the important part of angiotensin II (Ang II) in the pathogenesis of hypertension and other cardiovascular ailments is broadly explored. The RAS has for quite some time been perceived to assume a significant part in the direction of pulse and electrolyte adjust. It is an enzymatic course response (Guo et al., 2006). The preparing plan starts with the change of angiotensinogen (AGT) to angiotensin I (Ang I) with the help of renin. Ang I has practically no natural action however is changed over crosswise over vascular beds, especially in the lungs, to the octapeptideAng II. Angiotensin changing over compound (ACE) plays a major role in the plan; it catalyzes the C-terminal dipeptide (L histidyl-L-leucine) from the idle decapeptideAng I, to create strong vasoconstrictor Ang II (Ferrario et al., 2005). The customary view that Ang II is the key result of RAS has been addressed with the disclosure of angiotensin changing over chemical 2 (ACE2), also as developing confirmation for a physiological part for Ang (1 to 7). ACE2 changes over Ang I to Ang 1to 9, which can be further hydrolyzed by ACE to frame Ang 1-7. ACE2 additionally enhances the age of Ang 1 to 7 by expulsion of a COOH-terminal amino corrosive from Ang II. The putative result of ACE2, Ang 1 to 7, is a strong vasodilator and intervenes impacts inverse to those of Ang II in a few tissues (Lieberman, Krauthammer, & Sastre, 1986).

The balance between Angiotensin II and Ang (1-7), reflecting ACE and ACE2 activities, is to be considered as physiologically huge proportion. Over reactivity of the renin-angiotensin system (RAS) has been distinguished as a vital determinant that is involved in

the etiology of diabetes, heart disoders and hence speaks to a noteworthy focus for treatment (Mokdad et al., 2001). Ang II is the fundamental effector hormone of the renin-angiotensin system (RAS), which directs blood volume, blood vessel weight, and heart and vascular function. Ang II ties to two distinct receptors, type I (AT1) and type II (AT2). The Ang AT1 receptor intercedes the vast majority of the known physiological and pathophysiological activities of Angiotensin II in renal, cardiovascular and neuronal systems. Ang II likewise ties to AT1 and AT2 receptors, inciting a counter-administrative vasodilatation that is generally interceded by bradykinins (BK) and nitric oxide (NO) (Vickers et al., 2002).

2.6 Angiotensin Converting Enzyme (ACE) Pathway

ACE was discovered in plasma in 1956 by Leonard T. Skeggs. ACE is additionally present in different organs, for example, kidney, heart, pancreas, and cerebrum. ACE is a monomeric, layer bound, zinc and chloride subordinate di-peptidyl carboxypeptidase (Tiret et al., 1992). ACE is a critical helpful focus for the administration of hypertension dusring Diabetes. Interruption of ACE that diminish the development of Angiotensin II have been exceptionally fruitful in the administration of pretension in type 2 diabetes. Pathological activation of tissue ACE with resulting increase in nearby Ang II produces pernicious consequences for the kidney and the heart during organ remodelling, ischemic damage and restenosis (Valitutti, Müller, Cella, Padovan, & Lanzavecchia, 1995). The kidney, under the control of Ang II (delivered by ACE) and aldosterone, keeps up the electrolyte balance in the body. The present investigation of expanded ACE expression uncovered that higher ACE was related with an increased diabetic pathology, as far as renal capacity and circulatory strain (Agerholm-Larsen, Nordestgaard, & Tybjærg-Hansen, 2000).

2.7 Angiotensin-II Converting Enzyme (ACE-II)in Diabetes

Angiotensin Convertig Enzyme (ACE), a dipeptidyl carboxypeptidase, is a major compound in the renin– angiotensin system (RAS); it changes over the dormant decapeptide, Ang I to the dynamic octapeptide and intense vasoconstrictor Ang II and the vasodilator bradykinin is inactivated(Feng et al., 2002). Angiotensin II is thought to be in charge of the vast majority of the pathophysiological and physiological impacts of the RAS, and ACE geneinhibitors that lessen the production of Ang II have been exceptionally fruitful in the administration of hypertension, are standard treatment following myocardial infarction for a delayed heart failure development, and diminish the rate of acquiring of renal disorders (Lee & Tsai, 2002). Recently, the established perspective of the RAS is tested by the finding of the ACE2 enzyme, notwithstanding the expanding knowledge that various angiotensin peptides other than A It is anticipated that heart disorders will be the main source of death by the year 2020, although the current ways to deal with obstruct the RAS are of major benefit around there, it is presently certain that different pathways and catalysts inside the RAS can adjust its fundamental effector, Angiotensin II (Ritz & Orth, 1999).

2.8 ACE-II Structure and Function

ACE2 structure is the first human homologue of ACE known to mankind, and was cloned from a human cardiac failure library of cDNA. Investigation of the genomic grouping of ACE2 has uncovered that the quality consists ofeighteen exons and maps to chromosomal

location Xp22 (Hubert, Houot, Corvol, & Soubrier, 1991). The full-length, human ACE2 cDNA express a protein of 805 amino acids that has a homology of 42% with the N-terminal catalytic domain of ACE, and a water retentive locale close to the C-end. Like ACE, ACE II is anticipated to have the geography of a type 1 layer protein, with the catalytic space on the outer surface (Tiret et al., 1992).

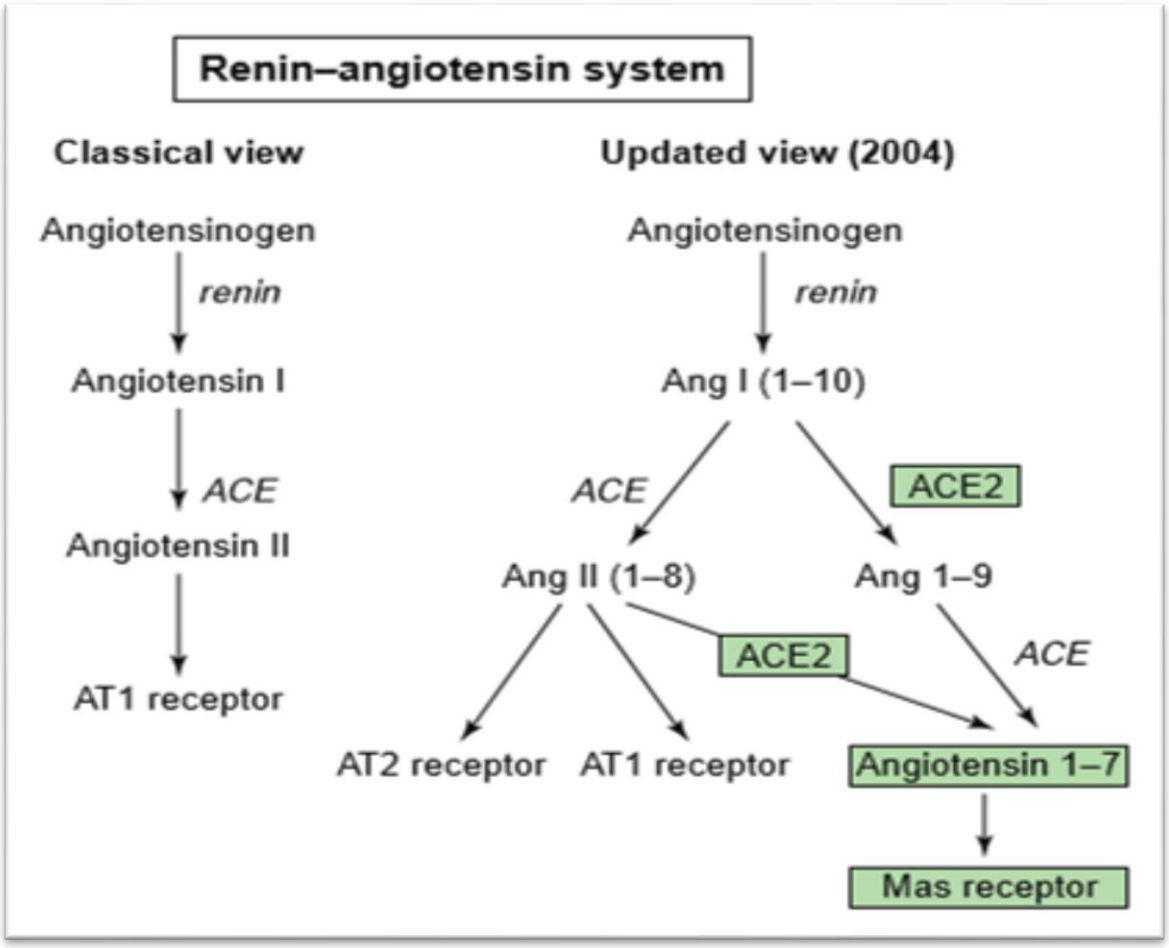


Figure 2.2: Rennin-Angiotensin Pathway – Function of ACE in the classical and updated studies

Contrary to somatic ACE, ACE2 has just a single dynamic enzymatic site and actions as a carboxypeptidase instead of a dipeptidyl carboxypeptidase. Accordingly, ACE II expels a solitary C-terminal Leu deposit from Ang I to create Ang 1 to 9, a peptide which has no

function to be kown. Despite the fact that ACE2 was depicted at first for its working to create Ang 1– 9 from Ang I, it likewise debases Ang II to the organically dynamic peptide, Ang 1 to 7 (Arpagaus et al., 1994). In reality, in vitro studies show that the enhancing efficiency of ACE2 for Ang II is 400-crease more noteworthy than for Angiotensin I, indicating that the major role for ACE2 is the transformation of Ang II to Ang 1 to 7. The potential part of Ang 1– 7 as a cardio-protective peptide with vasodilator, hostile to development and against spreading activities has been perceived generally. Taken together, the information recommend that ACE2 may function to restrict the vasoconstrictor activity of Ang II via its inactivation, notwithstanding balancing the activities of Ang II via the arrangement of the agonist, Ang 1 to 7. Late investigations have identified the G-protein-coupled receptor encoded by the MAS1 protooncogene, Mas, as the receptor for Ang 1– 7 (Arpagaus et al., 1994; Tiret et al., 1992). A quick action of ACE II has as of late been detected and portrayed; ACE2 is a practical receptor for coronaviruses, including the coronavirus that causes extreme intense respiratory disorder, and is associated with interceding infection section and cell combination. Despite the fact that not straight forwardly pertinent to cardiovascular capacity, this would demonstrate that the RAS along with ACE II has numerous parts in pathology and different physiological states (A. J. Scheen, 2004).

2.9 RAS Inhibition and Diabetes Prevention

Evidence recommends that the RAS may add to an impaired insulin discharge. In rodents, Ang II diminished pancreatic islet blood stream, which could prompt a decreased insulin discharge by the beta-cells, while RAS bar improved pancreatic blood stream (A. Scheen,

2004). Additionally, the foundational RAS, which is known for its traditional consequences for circulatory strain, electrolyte and liquid homeostasis, RAS parts have been recognized in numerous tissues, including the kidney, heart, mind, nerve filaments, conceptive organs, veins, liver, skeletal muscle, AT, and pancreas (Gross et al., 2005). Along these lines, it might well be that the pancreatic RAS additionally specifically influences beta-cell capacity and mass. In fact, in vitro and in vivo thinks about performed in mice have demonstrated that the RAS prompted islet fibrosis, oxidative pressure, and weakened insulin secretion, while RAS barricade with an ACE improved islet morphology and work and expanded glucose tolerance (Liu, 2007).

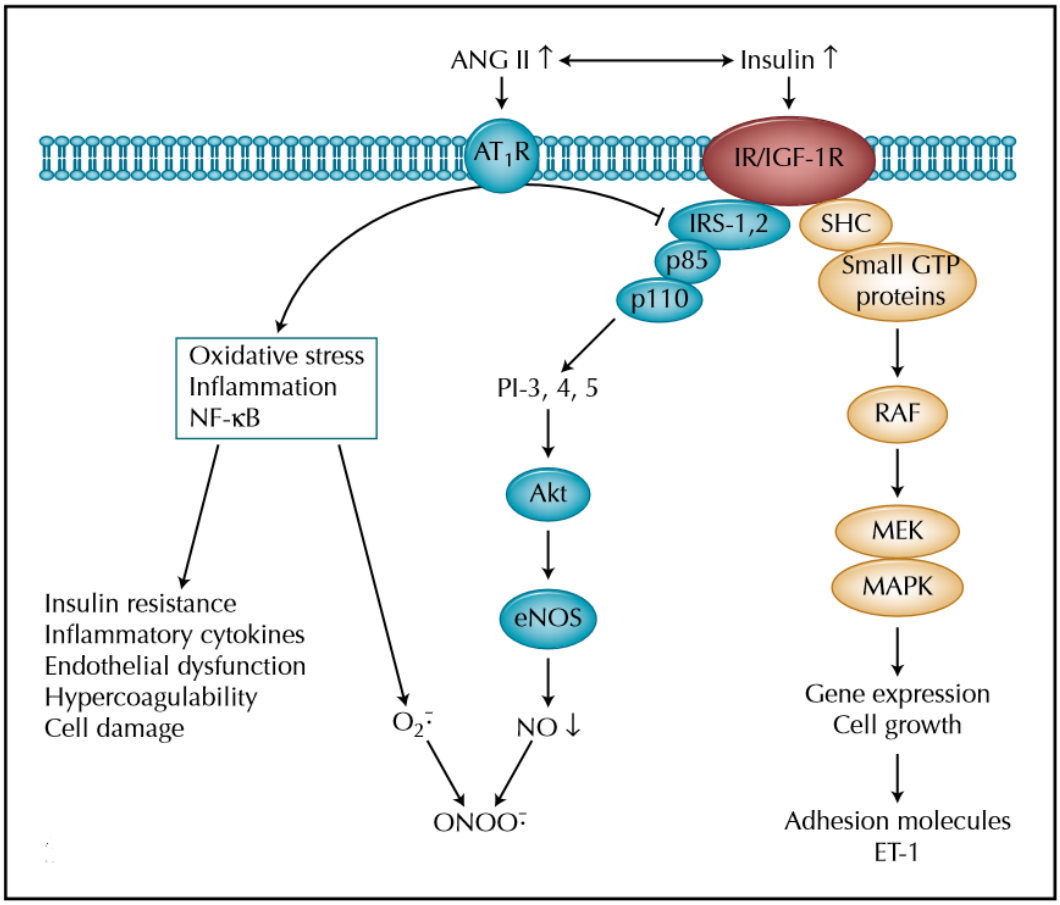


Figure 2.3: Insulin Resistant State – Angiotensin-II working

RAS activation expands ANG II, which cross-talks with the insulin signaling pathway and causes insulin resistance. This RAS enactment prompts irritation by means of the NF-LB provocative pathway. The contemporaneous enactment of the RAS, insulin protection, and irritation may cross-stimulate each other and coordinately add to endothelial brokenness, vascular damage, and quickened atherosclerosis. Along these lines, it is clinically critical to tame the RAS in patients with type 2 diabetes (Rakugi & Ogihara, 2005). The cardiovascular and renal advantages of RAS inhibition are most likely auxiliary to the joined impacts of brought down pulse, diminished aggravation and oxidative pressure, and enhanced endothelial capacity and insulin sensitivity (Rakugi & Ogihara, 2005; Ramalingam et al., 2017).

Chapter 3

Materials & Methodology

3.1 Ascertainment of Study Subjects

The case control study was planned in a case control manner. This study is approved by Ethical Review Board of ASAB, NUST. The study subjects were Type 2 Diabetes Mellitus patients from three different hospitals; Military Hospital, Rawalpindi, Holy Family Hospital, Rawalpindi and KRL Hospital Islamabad.

Informed Consent was signed by each patient; two groups of samples were obtained; one group consisted of 150 blood samples from the type 2 diabetic patients and the second group consisted of 50 blood samples from healthy individuals to be used as controls. A total of 5CC blood was drawn from patients of both genders between ages 30-60, having the Hba1c and BSR value higher than the normal range. Clinical diagnostic test values were noted down on the questionnaire for further analysis.. The blood samples were stored at -20◦C till further processed.

3.2 Materials

Glass wares and Micro-tips were cleaned, washed, autoclaved and oven dried at 60°C before using them. Following are the tables for different reagents used throughout the research. All the ingredients are in grams per liter of distilled water or as specified.

3.3 Reagents for DNA Extraction

Table 3.1: Solution A (Xu et al, 2004)

Sr.No.	Component	Molarity (mM)	Quantity (g/1000 ml)
1.	Sucrose	0.32	109.44
2.	Tris	10	1.21
3.	Magnesium Chloride	5	1.01
4.	Distilled Water		Upto 1000

Triton 100X (1% V/V) was added post autoclaving the solution

Table 3.2: Solution B (Xu et al, 2004)

Sr. No.	Component	Molarity (mM)	Quantity (g/1000 ml)
1.	Tris	10	1.21
2.	Sodium Chloride	400	23.37
3.	EDTA	2	0.58

Table 3.3: Solution C (Xu et al, 2004)

Sr. No.	Component	Quantity (µl)
1.	Phenol	400

Table 3.4: Solution D ((Xu et al, 2004)

Sr.No.	Component	Quantity (ml/500 ml)
1.	Chloroform	480
2.	Isoamyl Alcohol	20

Table 3.5: 20% SDS Solution

Sr. No.	Component	Quantity (g/100 ml)
1.	SDS	20
2.	Distilled Water	Upto 100 ml

Dissolved 20g of SDS in 100 ml of water

Table 3.6: 10X TBE Buffer

Sr.No.	Component	Quantity (g/1000 ml)
1.	Tris Base	108
2.	Boric Acid	55
3.	EDTA	7.5
4.	Deionized Water	Upto 1000 ml

The buffer was diluted upto 1000ml. White clumps were dissolved by using a magnetic stirrer on the vortex machine. **Note:** To make 1X TBE Buffer, 20ml TBE Buffer was dissolved into 180 ml of distilled water to make up a volume of 200ml

Table 3.7: 2% Agarose Gel

Sr.No.	Component	Quantity (g or µl/100 ml)
1.	1X TBE Buffer	100 ml
2.	Agarose	2g
3.	1X Ethidium Bromide	25 µl

Heated the agarose gel mixed with TBE Buffer, added the ethidium bromide on cooling and poured the mixture in the gel casting tray and let it cool till solid

3.4 Methodology

3.4.1 Sample Collection

The blood was drawn from the patients between the ages of 30-60 mainly females. The study was carried out on clinically assessed T2DM patients and the information linked to the disease was noted for the analytical study. 150 samples of blood were collected from the diabetic patients whereas 100 samples were collected from healthy individuals to be studies as controls. Consent forms were signed by the sample donors. The blood sample was collected in a properly labeled 10 ml EDTA tube. The name, age and identification number for each sample of the patient was noted down.

3.4.2 Genomic DNA Extraction

DNA Extraction was performed for each blood sample stored in an EDTA tube. The DNA Extraction was done by performing the following protocol:

Solution A, 500µl was added in a centrifuge tube along with the 500 µl blood sample, inverted 4-6 times and was then incubated at room temperature for 5 to10 minutes. Theeppendorf is then centrifuged for a minute at 13000 rpm in a micro-centrifuge. The supernatant was then discarded and the pallet was again suspended in 400 µl of Solution A

for lysing and again centrifuged for a minute at 13000 rpm. The supernatant was again wasted and the pallet was mixed with 400 µl of Solution B, 20 µl of SDS and 5 µl Proteinase K and was incubated overnight at 37◦C. After 24 hours, 500µl of freshly prepared solution C and solution D were added to the tube and were centrifuged for 10 minutes at 13000 rpm. The aqueous phase also called as the upper layer was collected in a new tube to which an equal quantity of solution D was added and it was centrifuged for 10 mins at 13000 rpm. The aqueous phase was again transferred to a new tube to which 55 µl of Sodium Acetate and an equal volume of isopropanol was added and the tube was inverted several times for DNA precipitation. The tube was then centrifuged for 10 mins at 13000 rpm and then the supernatant was wasted. 200 µl of 70% ethanol was then added to the tube and then again centrifuged for 7 minutes at 13000 rpm. The ethanol was then wasted and the pallet was dried for about an hour on a blotting paper, to which 200 µl TE Buffer was added and stored the DNA at 4◦C.

3.4.3 Angiotensin-II Converting Enzyme Gene (ACE) Primers

Following ACE Gene primers were used for the study

Seq. Name	Sequence	%GC Content	Product Size
ACE F	5’ CTGGAGACCACTCCCATCCTTTCT 3’	54.2	500bp
ACE R	5’ GATGTGGCCATCACATTCGTCAGAT 3’	48	200bp

Table 3.8: Reported primers with properties

3.4.4 Genotyping of ACE-II gene by ARMS PCR

All the apparatus used was autoclaved prior to usage, gloves and lab coat was worn throughout. The PCR reactions were performed on ice keeping it inside the laminar flow hood. Thermo Fischer PCR reagents were used.

3.4.5 Optimization of ACE-II Gene

To optimize the ACE-II gene, gradient PCR was performed for several days at different temperatures to find a specific temperature for the gene optimization.

3.4.6 PCR Profile

A reaction of 20 µl was prepared of each sample for the PCR experiment. The reagents were kept on ice throughout the execution of the protocol:

Reagent	Amount (µl)
PCR Water	9.8
dNTPs (2mM)	2
PCR Buffer	2
$MgCl_2$	2
ACE Forward Primer	1
ACE Reverse Primer	1
DNA Template	2
Taq Polymerase	0.2

Table 3.9: PCR Profile

3.4.7 PCR Cycle

The following PCR conditions were applied:

Process	Temperature	Time	Cycle #
Initial Denaturation	95◦C	10 minutes	
Denaturation	95◦C	30 seconds	35 cycles
Annealing	62.5◦C	30 seconds	
Extension	72◦C	30 seconds	
Final Extension	72◦C	10 minutes	
Storage	4◦C	∞	

Table 3.10: PCR Cycle

3.4.8 Amplification of ACE-II Gene

To study the association polymorphism, ARMS PCRwas carried out. All 150 DNA samples of diabetic patients and 100 DNA samples of healthy individuals were amplified with the ACE-II gene primers to observe genotyping. The reaction mixture of 20 µl volume was prepared.

The reaction master mix was prepared by adding 9.8 µl of PCR water, 2 µl of dNTPs, 2 µl of

Magnesium Chloride and 2 µl of PCR buffer (10X KCl buffer), whereas 1 µl of each primers (forward and reverse), 2 µl of DNA template and 0.2 µl of taq polymerase were added to each PCR tube individually.

The next step involves the processing the prepared reaction mixtures in the thermocycler. The required thermocycler conditions included the initial denaturation at 92◦C for 5 minutes, following the denaturation at 95◦C, annealing at 62.5◦C and extension at 72◦C for 35 cycles after which the final extension for 10 minutes is performed at 72◦C and the products are stored at 4◦C till further processed for observation.

3.4.9 Agarose Gel Electrophoresis

For analyzing the PCR products 2% agarose gel is prepared; 4g of Agar is added to 200 ml of 1X TBE buffer and is heated until the agarose is dissolved completely, giving a transparent appearance. It is then set to cool a little, to which 500 µl of Ethidium Bromide is added from the stock. The prepared mixture is then poured into a gel casting tray having the combs set for the wells and left to cool until solid. On solidification the gel was run with 10 µl PCR product and 2 µl of Bromophenol Blue loading dye. A 1000bp ladder was also run in a separate well on the same gel for analyzing the product size. The PCR product and ladder loaded gel is then placed in 1x TBE running buffer at 60V for one hour. The results obtained were then observed under UV light in a gel doc system.

3.4.10Association Studies for ACE Gene Polymorphism

Association studies to analyze ACE gene Polymorphism was done. Comparison between

controls and diabetic patients were made according to th respective alleles. Overall analysis was done through SPSS software.

Chapter 4

Results

Experimental study was carried out for ACE gene association analysis I and D polymorphs in diabetic patients from Pakistani population. A study group of 200 individuals consisting of 150 diabetic patients and 50 healthy individuals was taken under consideration. Studies were carried out on the DNA extracted from the blood samples of the individuals selected for research study. Statistical analysis of resulting data was carried out. There was a significant difference in groups reporting the three types of polymorphism i.e. II (Insertion) polymorph, I/D (Insertion-Deletion) polymorph and DD (Deletion) polymorph.

4.1 Genotyping of ACE gene

ACE gene, optimized at 62.5◦C was further genotyped. The T2DM samples were run along with the healthy controls, positive controls and negative controls and the results obtained were analyzed.

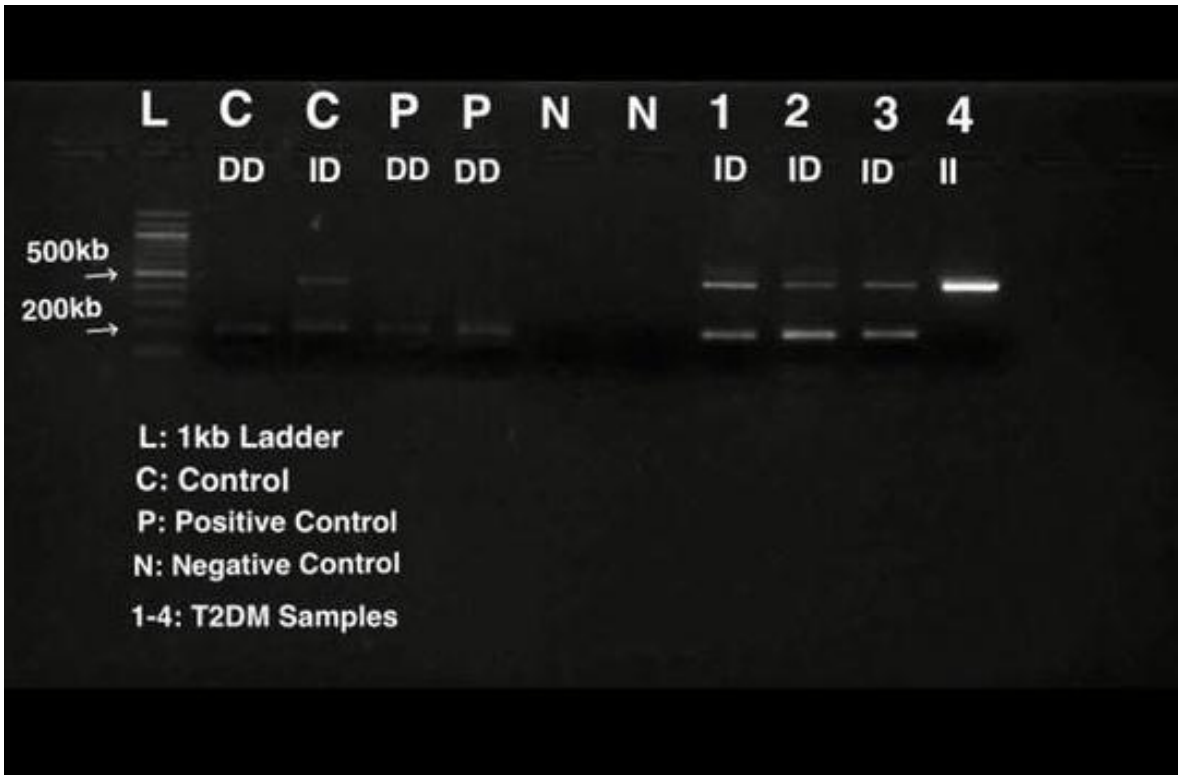


Figure 4.1: Gel Electrophoresis image of T2DM sample polymorphs along with the controls

4.2 Association Studies for ACE Gene Polymorphism

The analysis of the entire study included various parameters such as age, gender, weight, duration of disease and other related diseases. The data helped in generating the genotype frequency and the allele frequency of the Type 2 diabetes Mellitus patients as well as healthy control individuals. The frequency of ID (Insertion-Deletion Polymorph) was higher than the DD (Deletion Polymorph) and II (Insertion Polymorph).

Genotype	T2DM n = 150	Controls n = 50
II	12 (8%)	2 (4%)
ID	84 (56%)	18 (36%)
DD	51 (34%)	30 (60%)
Allele Frequency	**T2DM** **n = 300**	**Control** **n = 100**
I	108 (36%)	22 (22%)
D	186 (62%)	78 (78%)

Table 4.1: Genotypic and Allelic Frequencies of T2DM patients and healthy controls

4.3 Genotypic Frequency

The genotypic frequency of the ID polymorph was higher than the II and DD polymorphs in the diabeticpatients i.e. 56% in T2DM samples and moderate in healthy controls i.e. 36%.

Whereas that of II polymorph was the lowest in the both the study groups i.e. 8% in T2DM samples and 4% in healthy controls. The DD polymorph appeared moderately in the case of diabetic samples, 34% and appeared in a high rate of 60% in healthy controls.

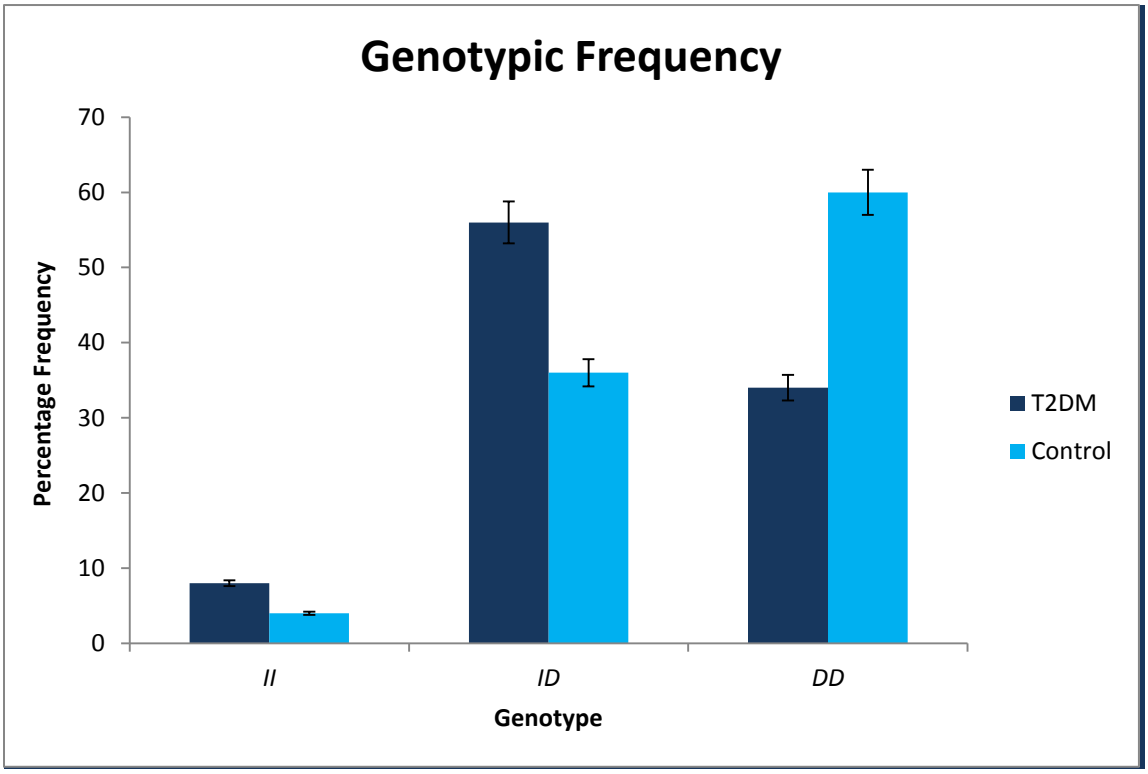


Figure 4.2: Genotypic Frequency of Polymorphs in T2DM and Control Study Group

4.4 Allele Frequency

The allele frequency for both the alleles (I and D) were calculated. The frequency of Insertion Allele (I) in diabetic samples was calculated as 36% and 22% in healthy controls. Whereas, the frequency of deletion allele (D) in diabetic patients was calculated as 62% and that in controls as 78%.

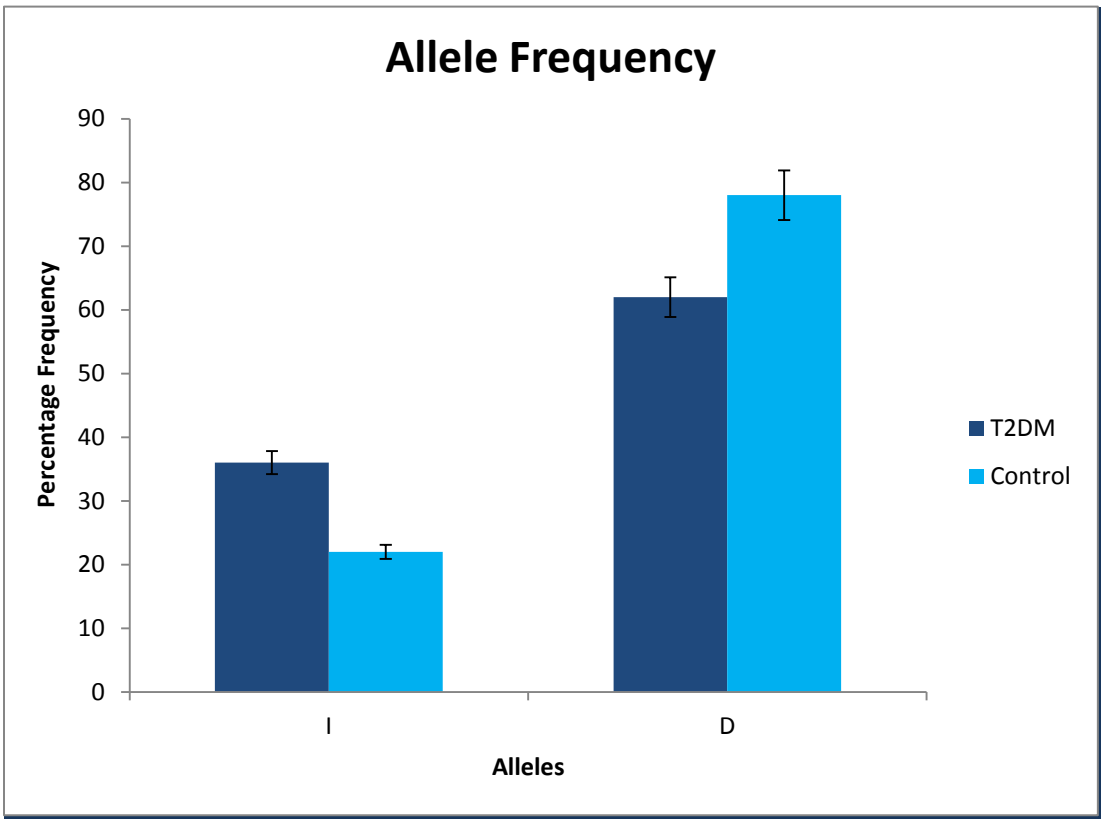


Figure 4.3: Allelic Frequency of I and D Alleles in T2DM and Control Study Group

4.5 Comparison between T2DM patients and Controls

The data of the T2DM patients and healthy individuals were compared statistically. The difference was seen between the genotypic frequencies of T2DM and healthy controls (χ^2=5.19 [p=0.23]). A significant association between the two study groups was observed under Odds Ratio (OR=2.0587, 95% [CI: 1.1002 - 3.8520], p=0.0239).

The difference in the polymorphs of both the diabetic and healthy control individuals was recorded in which a higher DD genotype frequency was observed in the controls than the diabetic individuals in which the ID genotype frequency was higher. In Allelic frequency

analysis the D allele frequency was higher than the I allele frequency in both the study groups.

4.6 Gender Based Association Studies for ACE Gene Polymorphism

Analysis on the basis of gender was also done. Genotypic frequency and Allelic frequency of both males and females were calculated to analyze the results.

Genotype	Male T2DM n = 20	Male Controls n = 25	Female T2DM n = 130	Female Control n = 25
II	0	0	12 (9%)	2 (8%)
ID	11 (55%)	7 (28%)	73 (56%)	11 (44%)
DD	9 (45%)	18 (72%)	42 (32%)	12 (48%)
Allele Frequency	**Male T2DM n = 40**	**Male Control n = 50**	**Female T2DM n = 260**	**Female Control n = 50**
I	11 (27.5%)	7 (14%)	97 (37%)	24 (30%)
D	29 (72.5%)	43 (86%)	157 (60%)	35 (70%)

Table 4.2: Gender Based Genotypic and Allelic Frequencies of T2DM patients and healthy controls

4.7 Genotypic Frequency

There is no significant difference between the genotypic values of males and females in both

the study groups of diabetic patients and healthy controls. The frequencies of the gender based and overall analysis are statistically the same i.e ID polymorphs are higher than DD and II polymorphs inT2DM whereas DD polymorphs are more in the healthy controls for both males and females.

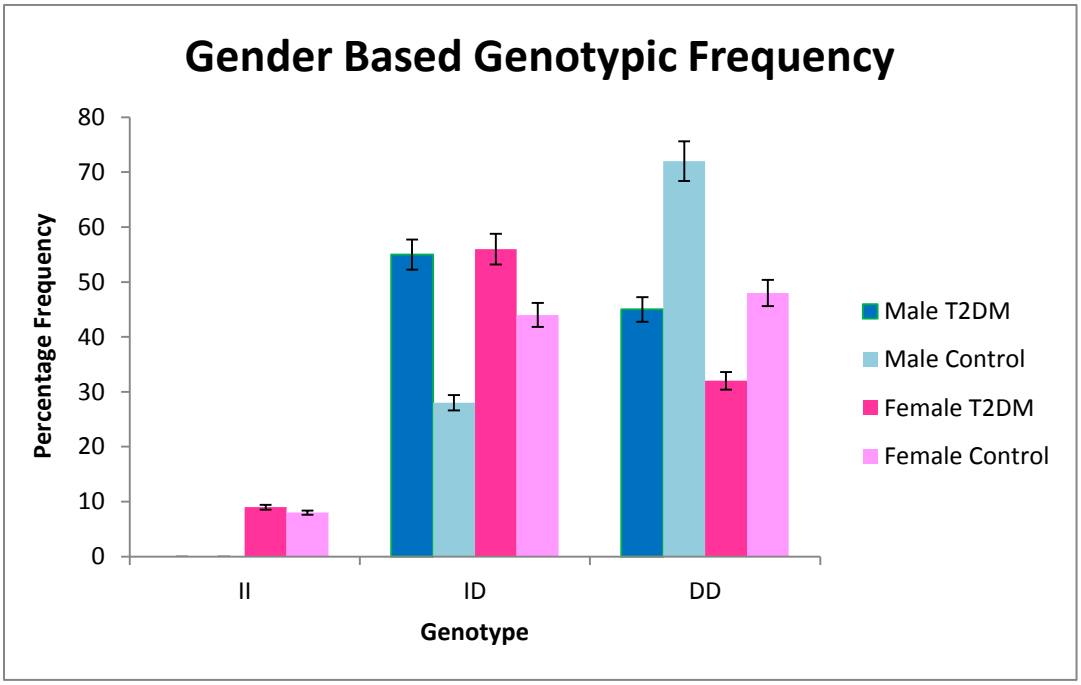


Figure 4.4: Gender Based Genotypic Frequency of Polymorphs in T2DM and Control Study Group

4.8 Allelic Frequency

In both males and females, the D allele frequency is higher than the I allele frequency in both study groups just as analyzed in the overall analysis.

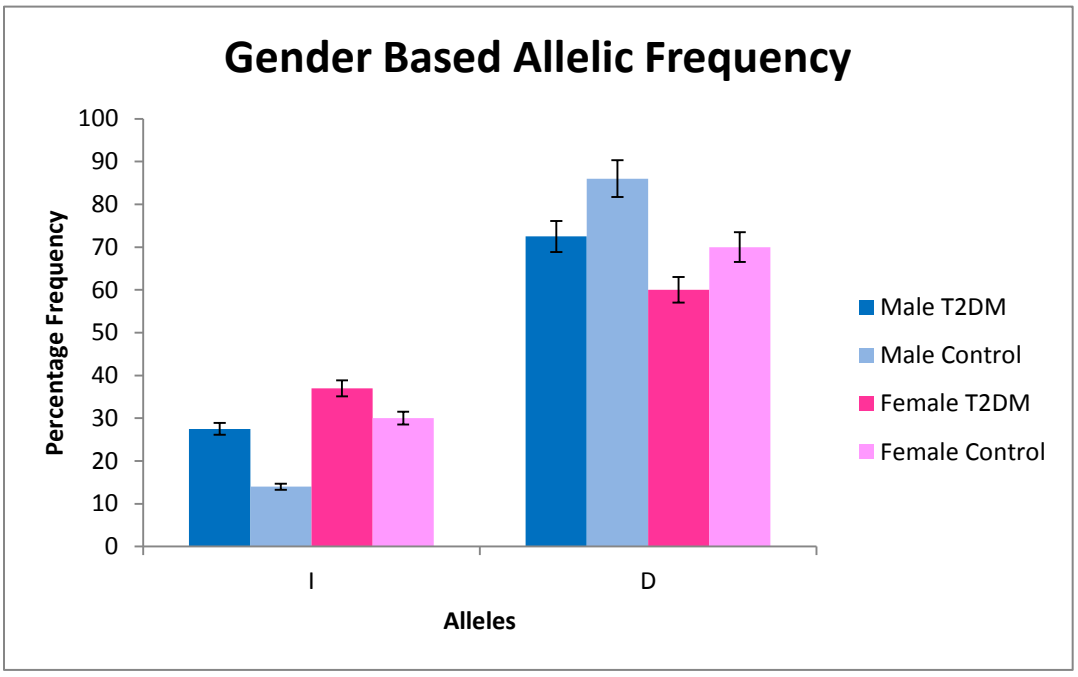


Figure 4.5: Gender Based Allelic Frequency of I and D Alleles in T2DM and Control Study Group

4.9 Gender Based Comparison

Association between ACE I/D polymorphisms and T2DM was studied on the basis of gender. As I the general analysis, there was no significant difference in the gender based studies. The polymorphic frequencies were calculated to be the same in both males and females and no gender allele frequency differentiation was observed in either of the study group. In the T2DM male individuals, the frequency of I/D polymorphs was observed higher than the DD and II polymorphs and same was calculated in the female individual. Whereas in the control samples, the DD genotype had a higher frequency than the I/D and II genotype in both males and females.

However, the D allele frequency was studied to be higher in both the study groups, i.e.

Diabetic patients and healthy controls of both genders. Therefore, both genders carry equal ACE gene polymorphs and there is no allele differences on the basis of gender parameter.

Chapter 5

Discussion

Type 2 Diabetes Mellitus (T2DM) is a metabolic dysfunction, the progression of which is inspired by a number of genetic, hormonal and environmental factors (Inzucchi et al., 2015). Various features like family history, gender, age, obesity and ethnicity play a vital role in causing a higher risk of developing type 2 diabetes mellitus (Marso et al., 2016b). It's a polygenic disorder, highly affected by the genetics for which various genes have been brought under study. Of all the genes studied in regard to type 2 diabetes mellitus, 5%-10% have been noted to have been influencing T2DM in various individuals depending upon their specific parameters (Giorda et al., 2017). Angiotensin Converting Enzyme gene (ACE) has a direct impact on the development of T2DM thus ACE inhibitors have been developed to reduce the risk of T2DM in a certain population (Shi et al., 2015).

The hormone regulating system, the Renin Angiotensin System (RAS) has a major role in maintaining the fluid balance and keeping the blood pressure of the body in control (Rossi et al., 2018). In the regulatory pathway, Angiotensin Converting Enzyme converts angiotensin I to angiotensin II. Angiotensin II being the potent vasoactive peptide, cause the blood pressure constriction thus directly affecting the blood pressure (Rice et al., 2014). Angiotensin II production also controls the hormonal activity releasing aldosterone that regulates the absorption of salts (NaCl) and water in the kidneys, creating an imbalance. Any regulation imbalance therefore leads to harmful effects including T2DM (Borgo et al., 2015).

The study aimed to find the association between ACE polymorphism in relation to type 2 diabetes mellitus. 150 blood samples of T2DM patients were collected and were experimented for ACE polymorphism through ARMS PCR after optimizing the primers at 62.5◦C. The results obtained were observed through gel electrophoresis on a 2% agarose gel under UV light. The gel images showed associations at to different sizes; 500bp and 200bp. The normal sequence without any insertion polymorphism was considered to be DD (deletion) polymorphism, showing just a 200bp size product. 34% of the T2DM patients showed the DD polymorphism and 60% results from the healthy control group were acquired thus highlighting the DD polymorph as the correct form. Both I and D polymorphism was observed in 56% of the diabetic patients and 36% of the control group, showing a product of two different sizes i.e. 500bp and 200bp. This showed the I/D (insertion-deletion) polymorphism to be in a higher number in the diabetic individuals. Whereas the insertion polymorphism, which has a product size of 500bp, was observed the least of all the T2DM and healthy control samples; 8% in T2DM patients and 4% in healthy controls thus depicting the least involvement in the T2DM development.

Strong association of D allele was observed in the overall study. In T2DM, the acquiring of a higher percentage of DD (deletion) polymorph observed shows an authentic relationship of deletion polymorphism to be present in less affected individuals, genetically (Al-Jafari, Daoud, & Ataya, 2017). However, the T2DM patients having DD (deletion) polymorph also shows the high association of D allele frequency to T2DM (Motawi, Shaker, Shahin, & Ahmed, 2016). The D allele frequency in T2DM group was calculated to be 62% and in that of a control group to be 78% which depicts the importance of deletion polymorph more

significantly.

The insertion-deletion polymorphism was observed more in the T2DM group than the control group which contributed to increase the I and D allele frequency in both study groups. The involvement of a 500bp sequence to a normal deletion sequence disturbs the normal sequence form thus causing insertion-deletion I/D polymorphism and increasing the risk of acquiring T2DM. Majority of the diabetic individuals have been studied to have I/D polymorphism present in them, also showing the genetically acquired T2DM running in the families thus showing a significant difference between both the study groups.

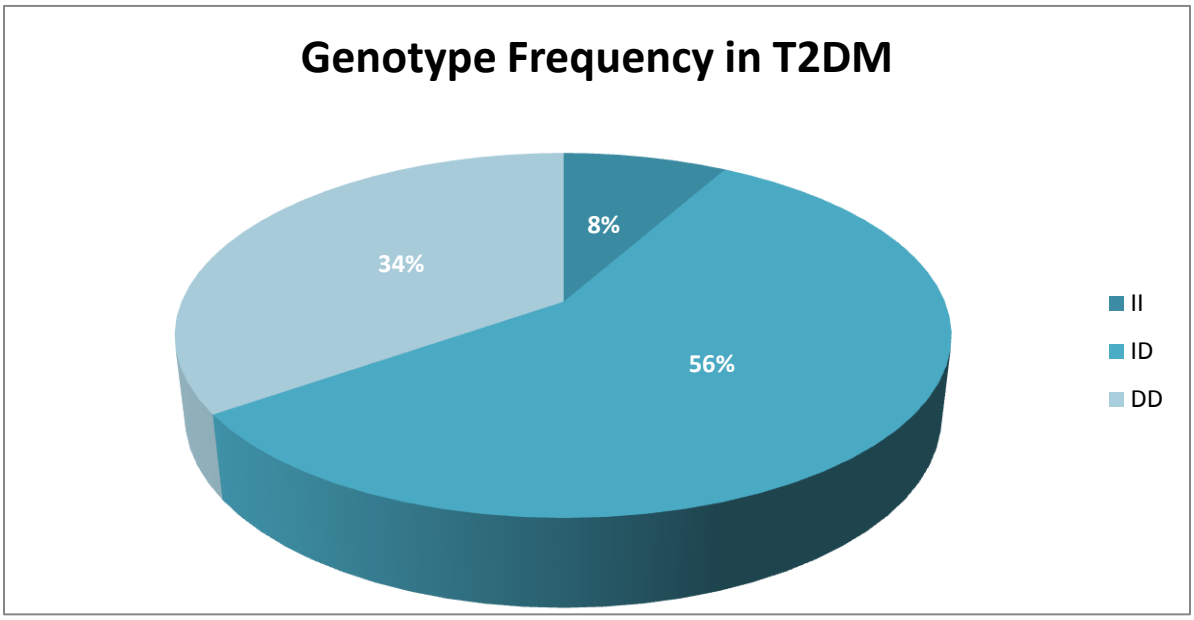


Figure 5.1: Observed Genotype Frequency in T2DM

The insertion polymorphism was observed less in both T2DM and control group showing no significant difference between the two. But the marked difference observed was gender based as no male individual gave the insertion polymorphism results but the insertion-deletion

polymorph. Whereas the results acquired for insertion polymorphism only came from the females in both T2DM as well as the control group, therefore making it easier to conduct a gender based study in regarding to the ACE gene.

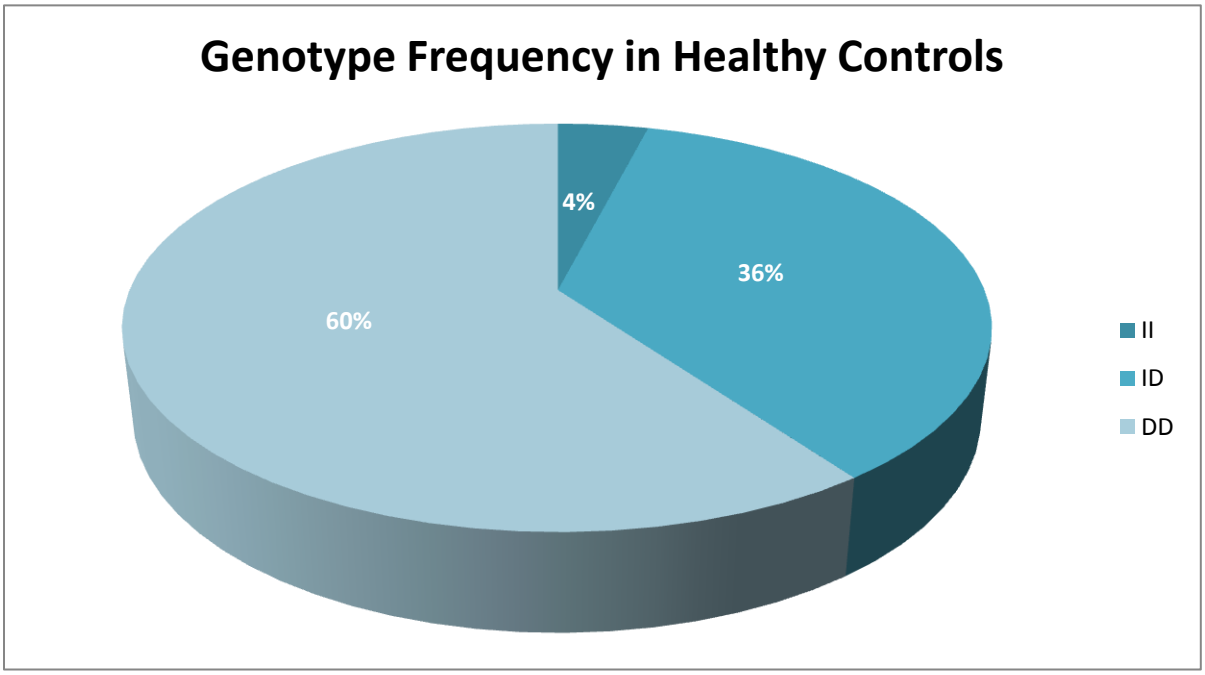


Figure 5.2: Observed Genotype Frequency in Healthy Controls

The overall allele frequency of both the alleles I and D was calculated in both T2DM and control group which showed a remarkable difference in the presence of both alleles in diabetic individuals as well as the healthy ones. Keeping in view all three genotypes, the I allele frequency was calculated to be 36% in T2DM study group and 22% in the healthy control group. Whereas the D allele showed a higher frequency of 62% in the T2DM individuals and 78% in the healthy individuals. To assess this, it is repeatedly observed that the deletion polymorphism has a significant importance in keeping the genotype normal. Therefore, ACE inhibitors can be used accordingly, depending upon the polymorphic study

of any individual to correct the genotype (He et al., 2016).

The p value observed in this regard was also significant and showed a marked difference between the two polymorphic alleles. Various studies have been observed which show the involvement of D allele in the development of T2DM (L. Li, Bai, & Sheline, 2016). In a recent study, the association of D allele of ACE gene was studied in a Malaysian population. This study was ought to identify I/D association polymorphism in relation to hypertension and T2DM. Results showed aa significant relationship of D allele with Hypertension as well as Type 2 diabetes mellitus (Normaznah, Azizah, Rosli, Saniah, & Kuak, 2016). Another study conducted similarly showed a strong link of D allele to diabetes in Caucasian and African population (Djogbénou et al., 2015) and in another study similar results were observed in an Arab population (Adughiman, Alashiekh, Gonzales, & Hale, 2015).

Hence from all the previous studies and research done on ACE polymorphism, and in regard to this study conducted, it is highly observable that the D allele shows high association of occurrence in Type 2 diabetes patients and the insertion-deletion polymorphism (I/D) shows patients of T2DM at a higher risk. Whereas the deletion polymorphism (DD) in a diabetic individual is one of the correct forms, showing no inheritance of diabetes that run in the families but other parameters apply. Therefore, the D allele frequency of ACE gene is significant for the study of Type 2 diabetes mellitus.

Conclusion

This is a unique study conducted in Pakistani Type 2 Diabetes Mellitus patient population for the association of insertion polymorphism with T2DM thus revealing the fact that Insertion-Deletion polymorphism (I/D) is strongly associated T2DM individuals and the deletion polymorphism (DD) is linked to individuals who are more on the healthy side. The D allele frequency is comparatively higher in T2DM and therefore this data can be helpful in inhibiting the disease by using allele specific ACE gene inhibitors, depending upon the genotypic frequency of the T2DM patient.

Chapter 6

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