ASSOCIATION ANALYSIS OF GENETIC POLYMORPHISMS *(rs2146323)* AND *(rs699947)* OF *VASCULAR ENDOTHELIAL GROWTH FACTOR-A (VEGF-A)* IN PATIENTS OF TYPE 2 DIABETES MELLITUS

BY

MINELLE HAMID

NUST ID **00000170961**

MS HEALTHCARE BIOTECHNOLOGY

SESSION 2016-2018

SUPERVISED BY

DR. ATTYA BHATTI

DEPARTMENT OF HEALTHCARE BIOTECHNOLOGY

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES,

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A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Masters of Science (MS)

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Examination Committee Members

1. Name: **Dr. Peter John** Signature: _____

2. Name: **Dr. Tougeer Ahmed** Signature: _____

3. Name: **Dr. Jahangir Sarwar Khan** Signature: _____

Supervisor's name: **Dr. Attya Bhatti** Signature: _____

Date: _____

Head of Department Date

Dr. Tougeer Ahmed
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date

COUNTERSINGED

Date: _____

Dean/Principal

Dr. Huda Ghayur Sanjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

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Name of Supervisor: **Dr. Attya Bhatti**

Date: _____

Signature: _____

HOD Healthcare Biotechnology: **Dr. Touqeer Ahmad**

Date: _____

Signature: _____

Principal ASAB: **Dr. Hussnain Janjua**

Date: _____

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_____

Minelle Hamid

To my dear S. S.

I owe this milestone to you.

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Minelle Hamid

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T2DM (Type 2 Diabetes Mellitus)

T1DM (Type 1 Diabetes Mellitus)

IGT (Impaired Glucose Tolerance)

IFG (Impaired Fasting Glucose)

HbA1c (Haemoglobin A1c)

ADA (American Diabetes Association)

BMI (Body Mass Index)

IDF (International Disease Federation)

OGTT (Oral Glucose Tolerance Test)

IAPP (Islet-Associated Amyloid Peptide)

GWASs (Genome Wide Association Studies)

SNPs (Single Nucleotide Polymorphisms)

GIP (Gastric Inhibitory Polypeptide)

GLP1 (Glucagon like Peptide 1)

ER (Endoplasmic Reticulum)

AGEs (Advanced Glycation end Products)

RCTs (Random Clinical Trials)

AGIs (α-Glucosidase Inhibitor)

AMPK (AMP-activated Protein Kinase)

PPARγ (Peroxisomes Proliferator-Activated Receptor-γ)

PGC1 (PPARγ Coativator 1)

GLUT (Glucose Transporter Type 4)

FFAs (Free Fatty Acids)

DPP4 (Dipeptidyl Peptidase 4)

SGLT2 (Sodium Glucose Co-Transporter-2)

DM (Diabetes Mellitus)

DME (Diabetic Macular Edema)

WHO (World Health Organization)

CSME (Clinically Significant Macular Edema)

ETDRS (Early Treatment Diabetic Retinopathy Study)

PRP (Panretinal Photocoagulation)

HIF-1α (Hypoxia-Inducible Factor)

VEGF (Vascular Endothelial Growth Factor)

RAVE (Rubeosis Anti-VEGF)

CRVO (Central Retinal Vein Occlusion)

DRS (Diabetic Retinopathy Study)

PDGF (Platelets-Derived Growth Factor)

IVTA (Intravitreal injections of Triamcinolone Acetonide)

VH (Vitreous Hemorrhage)

TRD (Tractional Retinal Detachments)

RAAS (Renin-Angiotensin-Aldosterone System)

PGF (Placental Growth Factor)

ORP 150 (Oxygen Regulated Protein)

PKC (Protein Kinase C)

MAP (Mitogen-Activated Proteins)

MMPs (Matrix Metalloproteinases)

AMD (Age-related Macular Degeneration)

siRNA (small interfering RNA)

Abstract

Type 2 Diabetes mellitus is a disease classified by low production of the hormone insulin and gradual loss of β-cells in the pancreas and chronic resistance to insulin in the body. The gene Vascular Endothelial Factor – A (VEGF-A) is up-regulated as a result of uncontrolled blood glucose levels. VEGF-A has been widely linked with the proliferation of Diabetic Retinopathy (DR) in the patients of DM. The study focuses on identifying the association and risk analysis of two of the most linked polymorphisms of VEGF-A *rs2146323* and *rs699947* with DM and its co-morbidity DR in a cohort of 106 patients with Type2 Diabetes mellitus in Pakistan. Allele specific PCR was employed to observe the association between polymorphisms and the possible disease pathogenesis. The study showed remarkable association between the polymorphism rs2146323 and T2DM. The heterozygous genotype CA showed susceptibility of developing DR in T2DM vs. control with OR= 3.99, 95% CI= (2.1-7.4) and P= <0.0001***. The chi square (x^2) value (46.1) and P= (<0.0001***) suggests a significant association between *rs2146323* with T2DM and a risk of developing DR. A relative association between the CA genotype of *rs699947* of VEGF-A, and T2DM vs. control was found with OR= 1.9, 95% CI= 1.05-3.52 and P= 0.03. The chi square (x^2) value (10.10) and P= (0.0064**) shows some association of T2DM co-morbidities with T2DM. The allelic difference in the distribution of alleles for both polymorphisms of VEGF-A studied show no significant difference in their distribution of alleles in T2DM and Controls. Further research with ample sample size is required to validate the findings of this research on a larger set of patient population.

Chapter 1

Introduction

1.1. Type 2 Diabetes mellitus (T2DM)

T2DM is a long-standing prevalent disease that is characterized by deregulation of breakdown of carbohydrates, proteins and lipids, which is a result of insulin resistance or impaired secretion of insulin, or a combination of both. T2DM is the most common (90% of all cases) than the other forms of diabetes, namely gestational diabetes and type 1 diabetes mellitus (T1DM). The progression and development of diabetes has been studied progressively, over the last few decades, owing to the fact that it has been deemed as the fastest growing epidemic in the world. Its major cause is consistent impairment of pancreatic β-cells which are responsible for Insulin secretion. This is usually preceded by insulin resistance in adipose tissues, skeletal muscles and liver. Manifestation of hyperglycemia is foreshadowed by prediabetes (Abdul-Ghani, Tripathy *et al*. 2006; DeFronzo 2009), which throws the individual at a higher risk of developing T2DM. Prediabetes can manifest itself as any of the following: Impaired fasting glucose levels, increased glycated haemoglobin A1c (HbA1c) or impaired glucose tolerance (IGT). IGT is characterized as the resistance of insulin in muscles and secretion of late second phase insulin after taking the meals. Whereas individuals with impaired first phase insulin secretion and hepatic insulin resistance are characterized with IFG levels (Abdul-Ghani, Tripathy *et al.* 2006). Prediabetics have HbA1c levels between; it is a composite group that is clinically diverse with respect to pathophysiology. Annually, some 3%-11%

prediabetics convert to diabetic individuals (Gerstein, Santaguida *et al*. 2007). The basal pathophysiology, disease development and clinical presentation of Diabetes mellitus (DM) can vary noticeably between individuals. And at times anomalous representation of symptoms of a patient can make precise classification of type 2 diabetes mellitus tough for the clinicians. Before clear-cut diagnosis the symptoms of DM may reveal themselves as asymptomatic for many patients, and for other patients the disease could manifest itself with diabetic ketoacidosis and or severe hyperglycaemia. Maturity onset diabetes of the young (Gardner and Tai 2012) and underlying autoimmune diabetes in adult individuals can be mistaken as T2DM (Hawa, Kolb *et al*. 2013). For such individuals the frequency and timings of screening tests depends on the risk factors involved (Evert, Boucher *et al*. 2014). Testing for T2DM in at risk individuals is important because as much as 30% of prediabetics remain undiagnosed. Certain lifestyle adjustments such as weight-loss and exercise may hail as a preventive step for individuals at a risk of developing diabetes. Anti-diabetics and prevention of obesity can also lower the possibility of developing T2DM (DeFronzo and Abdul-Ghani 2011). As per the recommendation of the American Diabetes Association (ADA) individuals at a high probability of developing T2DM (BMI ≥30 kg per m2 ; age ≤60; HbA1c >6.5%) should be treated with metformin (Nathan, Davidson *et al*. 2007). Pioglitazone (DeFronzo, Tripathy *et al*. 2011) and low dose administration of metformin and rosiglitazone (Zinman, Harris *et al*. 2010) can aid in preventing prediabetes to diabetes. A tailored lifestyle (weight loss and diet control programmes) to prevent diabetes could significantly decrease the chances of conversion to diabetes. T2DM is an intricate long-term disorder which requires care and constant medical charting, patient's disease awareness and self-management of high or low glucose levels, and several approaches to normalize the

level of glucose, blood pressure and lipid profile charting to combat or prevent minimization of any potential risk of associated long-term microvascular complications; neuropathy, retinopathy and nephropathy. And also macrovascular complications like stroke and heart attack (Pozzilli, David Leslie *et al*. 2010; Inzucchi, Bergenstal *et al*. 2012; Garber, Abrahamson *et al*. 2016). Effective preventive measures could pose as lifesaving. T2DM is a heterogenous disorder with a broad spectrum of Pathophysiological symptoms and conditions, with varying complication susceptibility and individual clinical and therapeutic response; it should be treated and viewed as such. Search for a true cure for T2DM in near future would require a deep study of the molecular aetiology of the disease and effective preventive measures to encounter the growing obesity epidemic and sedate lifestyle that is directly linked to the increasing number of individuals in the world diagnosed with diabetes or standing at the hairline margin of prediabetes and diabetes.

1.2. Epidemiology of Type 2 Diabetes Mellitus

1.2.1. Global Millstone of T2DM

T2DM and associated complications of the disease have a huge impact on global disability and mortality. In 2013, diabetes mellitus in all its forms, was classified as the 9th major cause of decreased expectation of life by a research conducted by the Global Burden of Disease Study (Naghavi, Abajobir *et al*. 2017). According to an estimate made in 2010, diabetes mellitus caused around 3.96 million deaths in adults aged twenty to seventy nine years during 2010, this accounted for 6.8% mortality rate globally (Roglic and Unwin 2010). An IDF (International Diabetes Federation) report expressed that during 2015, the estimated mortality increased to a whopping 5 million deaths because of T2DM and complications related

to it, this corresponds to the ratio of one death every six seconds (Cho, Shaw *et al*. 2018). Disability incidence due to diabetes complications has been observed to rise since 1990, particularly in people lying in age groups 15-70 years (Vos, Barber *et al*. 2015). The onslaught of diabetes mellitus commences years before the proper diagnosis of the disease. Estimatedly, 174.8 million or 45.8% of cases of all adult diabetic patients were undiagnosed globally (Beagley, Guariguata *et al*. 2014); patients with untreated diabetes and undiagnosed disease are at an elevated risk of developing complications than those who are getting appropriate diagnosis. To top it all, the medical expenditure is three times greater for the patients with T2DM than the general population without T2DM (Rubin, Altman *et al*. 1994). By an estimate of IDF, in 2015, 12% of global health expenditure i.e. US$673 billion was only spent on treating people suffering from T2DM and related complications (Cho, Shaw *et al*. 2018).

Worldwide, the number of people suffering from T2DM quadrupled between the years 1980 and 2014 (Collaboration 2016). It has been approximated that the frequency of T2DM will elevate to 20% in developed countries and up to in developing countries between the years 2010 (Shaw, Sicree *et al*. 2010). Asia stands out as the major area for prevailing increase in T2DM epidemic. India and China are the major hub of the global epidemic of diabetes (Vos, Barber *et al*. 2015). The T2DM epidemic in these countries is portrayed by lower younger age and Body Mass Index (BMI) of onset than in western populations (Kong, Xu *et al*. 2013). The striking incline in diabetics and prediabetics in these countries despite relatively lower index of obesity could be related to the fact that given the same body mass index (BMI), the general population of Asia has a higher percentage of less muscle mass to body fat mass and abdominal obesity (Chan, Malik *et al*. 2009). Additionally, scanty

nutritional value in utero and in early life years, and surplus nutrition in later years of life, can greatly contribute to hasten the curve of diabetes epidemic in such populations where nutrition transition is rapid and unbalanced, which includes reduced physical activity and unhealthy food habits. Diabetes is slightly more prevalent in men than in women (Federation 2013).

With better epidemiological studies our understanding of lifestyle, behavioral and biological risk factors involved in prevalence of T2DM has improved. Higher BMI levels that are linked with increased adiposity, is the major risk factor associated with T2DM (Fig 1).

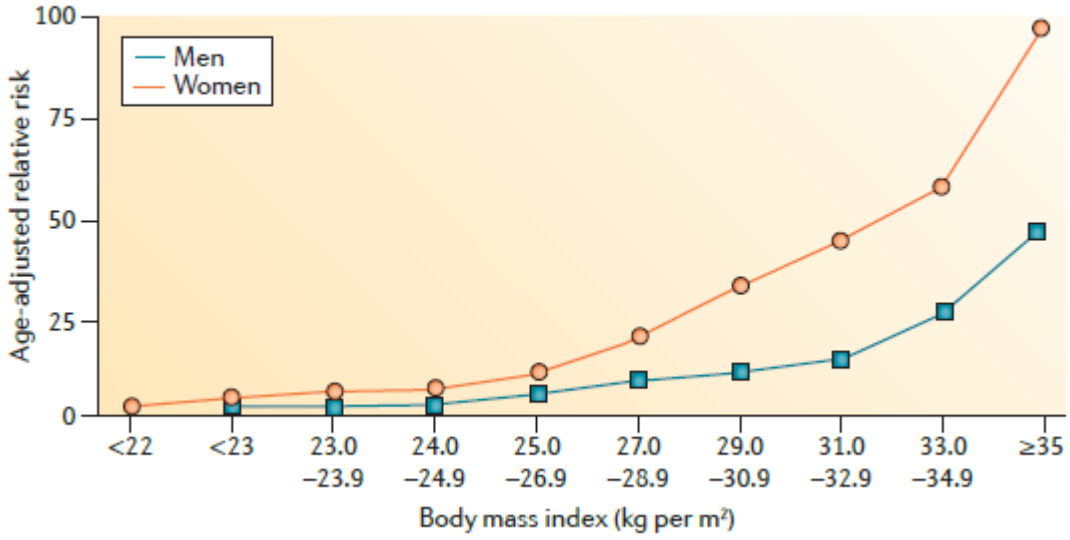


Figure 1: Association between T2DM and BMI.Chart obtained from REF (Type 2 diabetes mellitus, (DeFronzo, Ferrannini *et al*. 2015).

However, certain dietary components have been linked with reduced chances of developing T2DM, regardless of body weight. Together with large intake of green vegetables, coffee, nuts and whole grains; lower intake of processed and red meat, no

consumption of alcohol and sugary beverages and junk food, T2DM risk could be alleviated (Ley, Hamdy *et al*. 2014). One of the major behavioral risk factor is physical inactivity, which could be overcome with aerobic activity and resistance training (Grøntved, Rimm *et al*. 2012). Sedentary lifestyle puts people largely at risk for developing T2DM. Sitting for prolonged hours in front of television (Grøntved and Hu 2011), sleeping for both short hours (≤5 hr./night) and long hours (≥9 hr./night) (Cappuccio, D'Elia *et al*. 2010) and irregular rotating shift work (Pan, Schernhammer *et al*. 2011) are all possible risk factors. Cigarette smoking plays as a contributor for prevalence of T2DM to regardless of body weight and other risk factors (Hu 2011).

Childhood obesity stands out as the highest risk for the prevalence of T2DM in paediatric population (Bao, Tobias *et al*. 2014).

1.2.2. Prevalence of T2DM in Pakistan

It was researched in a community based study published in the start of 2019, which also stands out as the largest Diabetes Prevalence Survey in Pakistan that across the country the prevalence of T2DM is 16.98% and prediabetes stood at a 10.91% which has increased significantly than the ratio found in the only national survey of this kind conducted in the year 1999 (n=5433) using Oral Glucose Tolerance Test (OGTT) (Aamir, Ul-Haq *et al*. 2019). The ratio of persons at risk increased 2.68 times with a previous family history. This survey was conducted on the basis of HbA1c levels of patients and suspected patients. The test showed good sensitivity and specification for diagnosis of T2DM than OGTT. HbA1c is a simple yet an ideal test to be implied in community-based surveys in Pakistan, it is also less tedious.

In Pakistan, due to lack of sufficient primary care structure, a major chunk of the population is not subjected to basic screening for diabetes (Aamir, Ul-Haq *et al*. 2019). The prevalence of diabetes is inclining with each passing year. The high prevalence takes a toll on national economy and health budget as well as adds to the patient's own financial burden. According to the research conducted on diabetics in East Asia in 2012, 9.19% of females are prone to diabetes and males lie at 11.78% (Cho, Chen *et al*. 2012). Currently, Sindh has a preponderance of diabetics than any other province in Pakistan with 11.70% of females and 16.2% males (Meo, Asim *et al*. 2015).

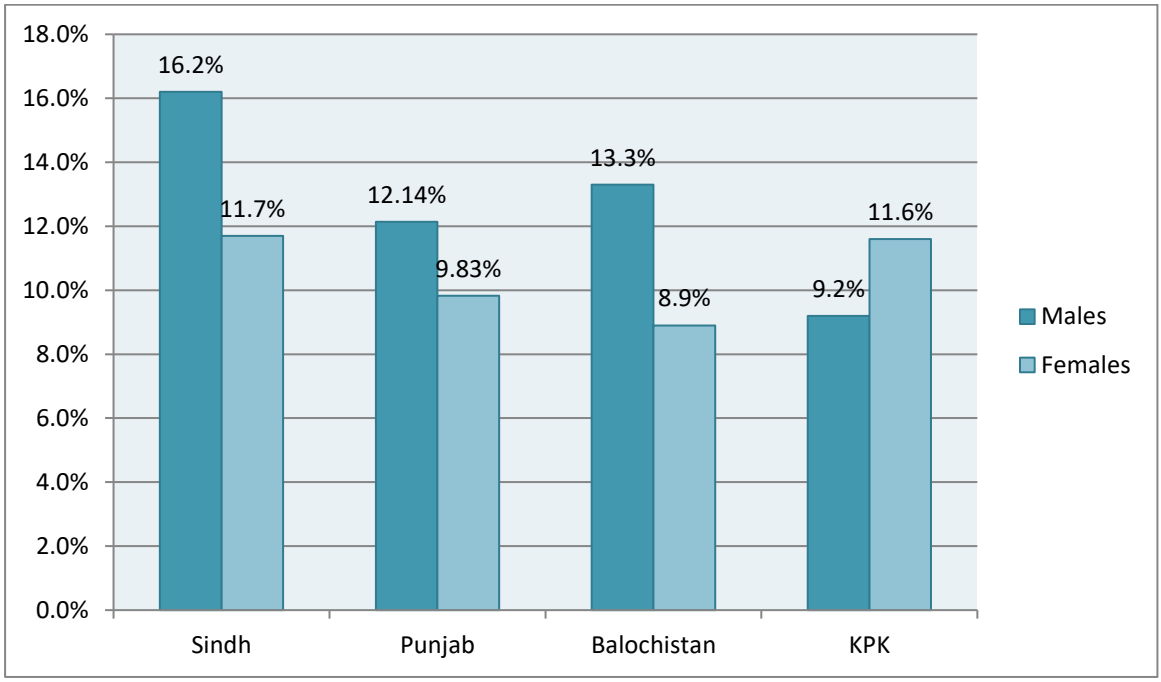


Figure 2: Gender Based Prevalence of T2DM in Pakistan (Meo, Zia *et al*. 2016)

Comprehensively, the ratio of diabetics in the non-rural areas of Pakistan is 14.81% in males and 10.30% in females. Pakistan should prudently include cost effective preventive measures in her National Health Policy to counter the rise of diabetic epidemic (Khan and King 1999).

1.3. T2DM Pathophysiology

Type 2 Diabetes mellitus is considered as a multifactorial disease with several genetic and environmental factors contributing to its pathogenesis. But majorly the Pathophysiological shifts are marked by insulin resistance, dysfunction of β-cells and chronic inflammation, all of which impedes management of blood glucose levels and ultimately leads to macro and microvascular complications. With the increase in age the production of insulin slows down due to the action of genes responsible for triggering diabetes or environmental factors. The β-cells of the body are significantly affected (Taylor 1999). IAPP (Islet-associated amyloid peptide) accumulation around or in islets of Langerhans is also responsible for insulin resistance (Nathan, Davidson *et al*. 2007). Hyperglycemia results due to excessive production of glucose by the liver and lower uptake of glucose in the surrounding tissues (Kahn 2003).

1.3.1. Genetic Determinants

T2DM is a disease characterized as heritable in families. The relative risk of prevalence of T2DM is higher for a particular person if the mother has the disease than if their father has (Groop, Forsblom *et al*. 1996). Establishing the genes responsible for triggering type 2 diabetes mellitus that is a polygenic disease has been challenging. In 2007, a breakthrough GWASs (genome wide association studies) noted common genetic variants linked with T2DM. TCF7L2 gene and the single nucleotide polymorphisms (SNPs) was most linked to T2DM (Grant, Thorleifsson *et al*. 2006) (Lyssenko, Lupi *et al*. 2007). Several other genes particularly their SNPs and loci have been linked with T2DM. They include genes like FTO, CDKN2A, CDKN2B, SLC30A8, GCKR, HHEX, CDKAL1, IGF2BP2 and others (Lyssenko, Lupi *et al*. 2007; Sladek, Rocheleau *et al*. 2007). It is notable here that these genetic

variants pose only a moderate risk by raising the disease developing chances by 10-20% and have been conserved during centenary of generations. Several non-diabetics also carry the risk alleles for T2DM and on average the frequency of an allele associated as a risk variant to T2DM is 54% (Sladek, Rocheleau *et al*. 2007).

1.3.2. β-cells Malfunctioning

Insulin resistance is the first possible detectable marker of T2DM in individuals who may possibly develop the disease (Gulli, Ferrannini *et al*. 1992; Martin, Warram *et al*. 1992). However, definitive T2DM does not appear if the β-cells secrete adequate amounts of Insulin to counter insulin resistance (De Jesus and Kulkarni 2014; Ferrannini and Mari 2014; Kahn, Cooper *et al*. 2014). The failure of β-cells to perform is an effect of several malfunctioning pathways including genetic abnormalities (Morris, Voight *et al*. 2012), lipotoxicity (Bays, Mandarino *et al*. 2004), reactive oxygen stress (Collins, Pi *et al*. 2012), hyper secretion of islet amyloid polypeptide (IAPP) (Ritzel, Meier *et al*. 2007), glucotoxicity (Bensellam, Laybutt *et al*. 2012), ageing 58, incretin hormone (gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP1)) deficiency or resistance (Madsbad 2014), stimulation of inflammatory cascades (DeFronzo 2010) and insulin resistance forcing β-cell stress (DeFronzo 2010).

1.3.3. Physiology of β-cells

Islets of Langerhans in humans constitute of α-cells (30%) responsible for secreting glucagon, pancreatic polypeptide producing cells (1%), β-cells (60%) and somatostatin producing δ-cells (10%) (Cabrera, Berman *et al*. 2006). In the islets, the β-cells work in sporadic subclusters which are connected via gap junction (Hodson, Mitchell *et al*. 2013). Every islet possesses some 100-500 μU insulin, this way the

entire pancreas has 10 days' worth of insulin supply (~ 1 million islets and of 0.9 g weight) for a normal adult (Brandhorst, Brandhorst *et al*. 1998). The proteins connexin provides with necessary communication between β-cells and other endocrine cells present in the islets and the other adhesion complexes in cells (Hodson, Mitchell *et al*. 2013). Hormones released in the blood stream can also influence the action of endocrine cells on each other. Non-hormonal products of the endocrine cells like Zinc and ATP and neurotransmitters also control the action of β-cells (Hodson, Mitchell *et al*. 2013).

1.3.4. Role of β-cells in T2DM

The β-cells mass was seen to be reduced by 30-40% in patients of T2DM than in patients without the T2DM, in their samples procured via post-mortem (Rahier, Guiot *et al*. 2008). However, morphometric measures are not always the same for individuals with T2DM or without it. The mass density of β-cells is established on feeding-fasting cycles, stress and exercise and on a minute-minute changes in order to maintain the normal blood glucose levels (Halban, Polonsky *et al*. 2014). An oral glucose challenge can trigger moderate to increased insulin secretion in T2DM except when the disease is left uncontrolled and untreated then absolute insulin secretion rate is declined. In advanced stage of T2DM β-cells functioning is viable but "stunned" and so can be activated to work through timely intervention (Ferrannini 2010).

1.3.5. Insulin Resistance

Insulin resistance is caused by obesity and sedate lifestyle which along with genetic tendencies (Morris, Voight *et al*. 2012) place stress on β-cell functioning, leading to a decline of insulin production and a loss of β-cell functioning (Shulman, Rothman *et al*. 1990) **.** Insulin resistance is not only apparent in liver and in muscles,

the tissues which play the role of major glucose disposal after the carbohydrate phosphorylation of IRS proteins, which obstructs phosphorylation of tyrosine that leads to insulin resistance (Bouzakri, Karlsson *et al*. 2006)**.** In several cases, IRS degradation by serine phosphorylation is responsible for adding to insulin resistance (Hiratani, Haruta *et al*. 2005). Serine phosphorylation is a multifactorial phenomenon that includes dysfunction of mitochondria, endoplasmic reticulum (ER) stress and inflammation and ectopic lipid accumulation.

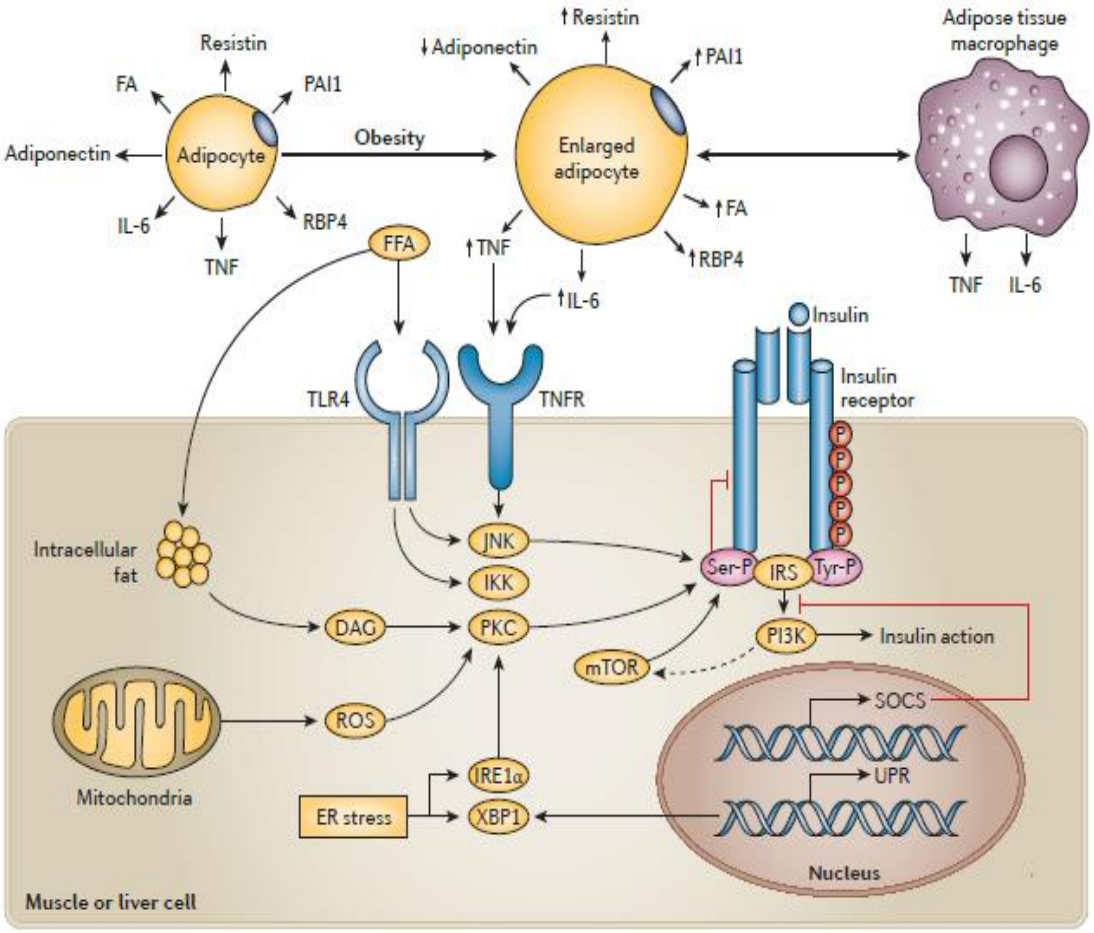


Figure 3: Insulin Resistance Mechanism| Free fatty acids (FFA), insulin-resistance initiating pro-inflammatory cytokines like tumour necrosis factor (TNF), interleukin-6 (IL-6) and resistin are released and produced in result of insulin resistance (also resultant of high metabolism of insulin receptor substrate (IRS) serine phosphorylation) and inflammation in Adipocytes. Increase in retinol-binding protein 4 (RBP4) may contribute to insulin resistance. Though, Plasminogen activator inhibitor 1 (PAI1) does not play a role in instigating insulin resistance but it is implicated in comorbidities of T2DM as obesity and heightened atherosclerosis. Resultantly, toxic lipid metabolites (diacylglycerol (DAG), acyl-CoAs and ceramides) accumulate in hepatocytes and myocytes, which hampers insulin signal transduction (IRS-phosphatidylinositol 3-kinase (PI3K) cascade) and activates inflammatory pathways such as JUN amino-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and IκB kinase (IKK), this further deregulates the signal transduction pathway of Insulin. DAG accumulation and activation of nuclear protein kinase C (PKC) which is caused by mitochondrial dysfunction and as well as high endoplasmic reticulum (ER) stress and production of reactive oxygen species (ROS) can further aggravate insulin resistance (Samuel and Shulman 2012). FA, fatty acids; SOCS, suppressors of cytokine signaling; TNFR, TNF receptor; mTOR, mammalian target of rapamycin; UPR, unfolded protein response; TLR4, toll-like receptor; XBP1, X box-binding protein (DeFronzo, Ferrannini *et al*. 2015)

1.4. Diabetic Complications

Prolonged Hyperglycaemia can cause severe diabetic microvascular complexities (Holman, Paul *et al*. 2008). Six major pathways activate the microvascular complications due to hyperglycaemia. Increased flux of polyol pathway, enhanced reactive oxygen species intracellularly (Brownlee 2005), PKC isoforms activation, high production of advanced glycation end products (AGEs), surge in expression of AGE receptors and increase in the flux of hexosamine (Brownlee 2005**).** Genetic determinants play a substantial role in incline towards microvascular complexities. T2DM also has a marked effect on macrovasculature. It increases the risk of stroke, myocardial infarction and peripheral vascular disease (Coutinho, Gerstein *et al*. 1999**).** Reactive oxygen species are responsible for triggering pro-inflammatory pathways, impairment of angiogenesis and activate epigenetic changes that can cause existing expression of inflammation causing genes whose expression may persist even after the glycaemia levels have been normalized. The molecular and biochemical pathways affecting microvasculature also contribute to macrovascular complexities (Giacco and Brownlee 2010).

1.5. T2DM Associated Risk Factors

Diabetes mellitus is not caused by one, but a number of factors. Some patterns have been found that indicate some plausible reasons that accentuate the onset of Diabetes mellitus. Some of these are appended below:

1.5.1. Obesity

Obesity is one of the main factors linked with Diabetes mellitus. Diabetes mellitus has also been dubbed as the sweet irony of the modern age. Excessive eating

of processed foods and junk food, without a lack of proper exercise has resulted in an exponential growth in Diabetes Mellitus over the years. To put things in perspective, excess body fat is associated with 100% of cases in Pima Indians and Pacific Islanders, 60–80% of cases in those of European and African descent, and 30% of cases in those of Chinese and Japanese origin (Gardner and Shoback 2011). A high waist-hip ratio is observed, for those who are not obese. This clearly shows the direct relation of obesity with the aforementioned disease. Europe has experienced a huge surge in Obesity and related diseases over the years. Recent Studies done by the National Health Service (NHS) shows the country is at the highest level of obesity in the continent, with 28.1% obese and 63.4% Overweight. Studies by the NHS also show that obesity is believed to account for 80-85% of the risk of developing type 2 diabetes and obese people are up to 80 times more likely to develop type 2 diabetes than those with a BMI of less than 22. Obesity refers to an individual having a BMI (Body Mass Index) of 30 and above; overweight Individuals' BMI lies in the range of 25-29.9. Most of the diabetes sufferers are overweight if not obese. The quality of quantity of the food and drink a person consumes has a direct impact on his/her health. Consumption of sugar sweetened drink in significant amounts is associated with increased risk (Malik, Popkin *et al*. 2010). Not all fats are harmful for the body. It was found that saturated and trans-fatty acids increase the risk and, monounsaturated and polyunsaturated decreasing the risk (Risérus, Willett *et al*. 2009).

1.5.2. Impaired Glucose Tolerance (IGT)

Impaired glucose tolerance refers to the pre-diabetes stage when blood glucose levels are beyond normal levels, but don't qualify for a Diabetes diagnosis. Impaired glucose tolerance exposes a person to a much greater risk of developing

cardiovascular diseases and Diabetes. It is associated with insulin resistance. IGT may precede type 2 diabetes mellitus by many years. The American Diabetes Association and the World Health Organization has described impaired glucose tolerance as two-hour glucose levels of 140 to 199 mg per dL (7.8 to 11.0 mmol/L) on the 75-g oral glucose tolerance test. Fasting levels of glucose are observed to be mildly elevated. Moreover, an individual is said to be influenced by IGT when they possess an intermediate level of raised glucose after 2 hrs., but overall less than the mark that would be characterized for T2DM. 10-15% of adults in the US suffer from impaired glucose tolerance erstwhile known as impaired fasting glucose (Rao, Disraeli *et al*. 2004).

1.5.3. Insulin Resistance

When Insulin is produced under insulin resistant conditions, it only creates matters worse as the cells are resistant to the insulin and it leads to high blood sugar. Insulin production is increased by the Beta cells in the pancreas, leading to high blood insulin levels. This often contributes to the development of type 2 Diabetes, as well as obesity and latent autoimmune diabetes of adults (Chiu, Tsai *et al*. 2007). A high intake of Fructose and other carbohydrates such as sugary drinks, leads to insulin rejection and directly contributes towards obesity. If we consumed such carbohydrates in excess, the insulin fails to absorb all of it and this results in elevated appetite (polyphagia), thirst (polydipsia) or urination (polyuria). However, a change in lifestyle including fasting and no-carbohydrate diet can reverse Insulin rejection.

1.5.4. Ethnic Background

Ethnic Background is found to have a direct correlation with prevalence of diabetes. South Asians are generally more prone to Diabetes, in addition to Chinese.

Africans are also at a greater risk of developing Diabetes. Numerous studies done on minorities and ethnic groups have shown that ethnicity has a direct impact on the risk of having diabetes. Various socio-economic factors and access to healthcare can also play a role in increasing or decreasing one's chance of being diagnosed with diabetes but it has been observed that even with equal access to health care, one's ethnicity has a direct linkage to risk of diabetes. A study carried out by the British Medical Journal came up with the following results:

i. People of South Asian descent are 6 times more likely to have type 2 diabetes
ii. African and Africa-Caribbean people are 3 times more likely to have type 2 diabetes

1.5.5. Gestational Diabetes

High blood sugar level during pregnancy is called gestational diabetes and these levels usually return to normal after giving birth. It is more common in the second half but can occur at any stage of pregnancy. During pregnancy the body has extra needs and in some cases the body cannot produce adequate amounts of Insulin to regulate the blood glucose levels. This type of diabetes can be a problem for the child and the mother during and after birth, but if timely detected and well treated it is easily manageable.

1.5.6. Sedentary lifestyle

A sedentary lifestyle is one of the drawbacks of the modern age. Lack of physical social interaction and birth of manufacturing processes has led to a sedentary lifestyle that encompasses lack of physical activity and exercise and obesity. According to the World Health Organization a sedentary lifestyle is one of the 10 leading causes of death and disability. In the United States alone, it accounted for 300,000 premature deaths. Controlling the Body Mass Index (BMI) is an effective

way to control diabetes. An exercise for 30 minutes a day and five days a week can prevent prediabetes developing into type 2 diabetes. The same amount of exercise can help reduce health risks and improve control of their condition, if a person already has type 2 diabetes.

1.5.7. Age

Type 2 Diabetes usually occurs in Middle-aged and older adults. According to the USA's Centre for Disease Control's 2017 National Diabetes Statistics Report, 2015 saw 1.6 million new cases of type 2 diabetes in older adults. The study also showed that adults aged 45 to 64 were the most at risk from diabetes. New cases of both type 1 and type 2 diabetes in people aged 18 years and above was as follows:

i. 18 to 44: 355,000 new cases
ii. 45 to 64: 809,000 new cases
iii. 65 and older: 366,000 new cases

Type 2 Diabetes was considered as a disease that affected adults, but the modern era has seen a rise in the number of young adults, and even children experiencing onset of type 2 diabetes. This is due to a sedentary lifestyle and large consumption of foods rich in carbohydrates and fats.

1.5.8. Abnormal Cholesterol and Triglycerides levels

It is believed by some researchers that surplus triglycerides increase the chances of diabetes as they lead to insulin resistance which further leads to high glucose levels. And when the body is ineffective in managing the high blood glucose levels, it leads to diabetes. They are also referred to as 'ugly fats'. They are derived from the diet and from carbohydrates in the body to fulfill the energy requirements.

When the body has fulfilled its present needs of energy, after a meal, the surplus fats are stored in adipose tissues, for later usage. They are also present in the blood. The excess amount of these gets deposited on the inner walls of the blood vessels, and results in various heart complications and stroke. As a standard, 150 mg/dl or above triglyceride in the blood is considered risky.

1.5.9. High Blood Pressure

High blood pressure complicates health of a person and when coupled with diabetes it has drastic effects. Their combination is thus lethal, and together they can increase the risk of a heart attack or stroke. They also increase in complications of kidneys, blood vessels and eyes (which can lead to blindness). Smoking also has a negative effect and increases chances of both hypertension and diabetes. Uncontrolled diabetes is one of the many risk factors for hypertension. People with diabetes should try to minimize these risks as far as possible, for example, by choosing a healthy lifestyle that includes physical exercise and a balanced diet.

1.6. Associated Complications

There are many complications that result due to a person suffering from diabetes. Proper medication and good management can reduce the under mentioned conditions.

1.6.1. Cardiovascular diseases

Serious heart complications can result from damaged blood vessels which are a direct consequence of raised blood sugar levels over a period of time, even if slightly raised. As the body is incapable to utilize all of this sugar properly, it tends to build up in the blood by sticking to the red blood cells. This build-up of sugar can

bruise the vessels carrying blood including vessels carrying blood to and from the heart, depriving it of oxygen and leaving them undernourished. In order to prevent damage to blood vessels and eventually the heart, HbA1c level should be as close to target as possible. Blood sugar, even if mildly above normal level, can put one at risk of developing diabetes.

1.6.2. Nerve Damage (Neuropathy)

One type of nerve damage that may happen as a result of diabetes is Diabetic Neuropathy. Raised levels of glucose (blood sugar) can adversely affect nerves throughout the body. Most of the times, the damage is apparent in feet and legs. Depending on the damaged nerves and extent of diabetic neuropathy, symptoms can be various ranging from pain and numbness in the legs and feet to problems with the urinary tract, digestive system, heart and blood vessels. While some individuals show mild symptoms, diabetic neuropathy can be quite trying and disabling in others. It is a prevailing and debilitating complication of diabetes. However, it can be averted, or its progression slowed down by control of blood sugar and following a healthful lifestyle.

1.6.3. Kidney Damage (Nephropathy)

Diabetes tends to injure minor blood vessels in the body. When the damage occurs in blood vessels of the kidneys, the kidneys cannot purify the blood properly. The body will retain more salt and water than it should, resulting in weight retention and swelling of ankles. Protein may be found in the urine and excretory material builds up in the blood. The nerves are also liable to be damaged by diabetes. This can pose as hazardous in emptying the bladder. The pressure built up due to a full bladder can be retained in and cause damage to the kidneys. High sugar level in urine provides

conditions for rapid bacterial growth which can cause infection in the bladder if not emptied for longer durations.

1.6.4. Eye Damage (Retinopathy)

Diabetic retinopathy is a vision threatening complication resulting from diabetes that takes a toll on the eyes. Caused by destruction of the blood vessels in the retina (light-sensitive tissue at the back of the eye). Initially diabetic retinopathy may cause no problem other than mellow weakness in sight. However, it can lead to eventual blindness. The condition can manifest in anyone who has type 1 or type 2 diabetes mellitus. The chances of developing this condition increases with the duration one is suffering from diabetes and high blood sugar levels remain uncontrolled.

1.6.5. Foot Damage

Nerve damage due to diabetes may result in diabetic neuropathy that can incite tingling and pain and make the patient lose sensation in the feet. Loss of feeling in the feet means that the patient will be unable to feel gravel inside their sock or a blister on their feet, that may result in blistering and cuts becoming infected. Another effect diabetes has is that it lowers the blood flow to the feet. Reduced blood flow to the limb extremities means that cuts and sores present there will be hard to mend. At times, a festering infection may never heal, getting worse and might advance into gangrene. Amputation of foot, toe, or part of the leg may become necessary if gangrene and foot ulcers do not mend with treatment. In order to save the patient's life and to prevent the infection from extending to the rest of the body, amputation may become necessary. Good foot hygiene and care is very important to hinder morbid infections and gangrene. Though rare but change of shape of feet can happen

as a result of nerve damage from diabetes as the likes of Charcot's foot. It initially may manifest as warmth, redness and swelling. Later, it can shift or break bones in the feet, which can result in the feet to morph into an odd shape, such as a "rocker bottom".

1.6.6. Skin Conditions

Including the skin, diabetes can adversely influence almost any element of the body. In fact, these may show as red flags that an individual may have diabetes. Luckily, if caught early, most skin conditions can be avoided or easily treated. These include skin problems that anyone can possess but diabetic individuals are more prone to developing them. These comprise of fungal infections, bacterial infections, and itching. Other dermatological ailments limited to diabetic people include diabetic dermopathy, diabetic blisters, necrobiosis lipoidica diabeticorum and eruptive xanthomatosis.

1.6.7. Hearing Impairment

According to a freshly introduced research funded by the National Institutes of Health (NIH), there are twice the number of adults with hearing loss who are diabetic compared to those who do not have diabetes. "Hearing loss may be an under-recognized complication of diabetes. As diabetes becomes more common, the disease may become a more significant contributor to hearing loss," was stated by the senior author Catherine Cowie, Ph.D., of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), suggesting hearing test for all people suffering from diabetes. "The study found a strong and consistent link between hearing impairment and diabetes using a number of different outcomes." The researchers found a marked increase in the incidence of hearing impairment in those with diabetes after

considering the outcomes of hearing tests given to a nationally representative sample of adults in America. The test measured subjects' ability to perceive low, mid, and high frequency of sounds in both ears. In all frequencies, a direct correlation between hearing loss and diabetes was apparent, with a stronger association in the high frequency range. Mild or greater hearing discordance of low- or middle-frequency sounds in the worse ear was about 21 percent in 399 adults with T2DM correlated to about 9 percent in 4,741 adults without diabetes. For higher frequency sounds, mild or greater hearing impairment in the worse ear was 54 percent in those with DM compared to 32 percent in those who did not have DM.

1.6.8. Alzheimer's Disease

Damage to blood vessels is one of the several complications caused by diabetes. Diabetes is considered a risk factor for vascular dementia. This type of dementia is a consequence of reduced or blocked blood flow to the brain causing brain damage. Brain changes that are hallmarks of both Alzheimer's disease and vascular dementia have been observed in people who have diabetes. Some researchers believe that both conditions fuel the damage caused by the other. Research is underway with an aim to grasp the link between Alzheimer's and diabetes. It is thought that the link may occur as a result of the complex ways that type 2 diabetes affects the ability of the brain and other body tissues to use sugar (glucose) and respond to insulin.

1.6.9. Depression

Most people with diabetes will not have depression at any given time. However, according to studies, people with diabetes are at a greater risk to suffer from depression than people without diabetes. A comprehensive explanation for why this is

true has not yet been reached. Daily management of diabetes can result in build-up of stress. All the extra work may tend to make the patient feel alone or apart from their friends and family. Diabetic complications such as nerve damage or one's inability to keep their blood sugar levels where one would like may tend to make them feel as if they have lost control of the diabetes. They may even feel frustrated and sad due to the existing tension between their doctor and themselves. Just like denial, depression can plunge them into a vicious cycle. It can block good diabetes self-care. Lack of energy and being depressed may make the daily routine task of regular blood sugar testing seem like too much. One may find it hard to keep a good diet if they cannot think straight due to being anxious. They may not feel like eating at all which will obviously affect their blood sugar levels. According to National Institute for Health and Care Excellence UK (NICE), people with diabetes and other chronic physical health problems are 3 times more likely to be diagnosed with depression than people without it. Depression can have a serious impact on a person's well-being, their ability and motivation to self-manage their condition. The most common psychiatric disorder witnessed in the diabetic community is none other than depression.

1.7. Diagnosis of T2DM

Traditionally, blood glucose level was the criteria for diagnosis of diabetes. Lately, HbA1c has been introduced to the list of diagnostics (Gillett 2009) as a consolidated standard of measuring long standing glycaemia. However, haemolysis, non-white race, alcoholism, old age, intake of high dietary fat, liver and kidney diseases and smoking cigarettes can affect HbA1c levels independent of glycaemia.

Several doctors acknowledge that diabetes should be characterized by the complexities involved; hyperglycaemia level linked with complications is varied according to the complication posed. Presently, maintained post-glucose-load-glucose, fasting glucose and HbA1c levels under the point linked with high risk of promoting diabetic retinopathy (Association 2010).

The diagnosis can be based on a raised random plasma glucose test (≥200mg per dl along with prime symptoms of hyperglycaemia), HbA1c (≥6.5%), fasting plasma glucose (≥126mg per dl after 8 hr. fast), 2 hr. post-glucose-load glucose level (≥200 mg per dl after 75 g oral glucose load) assessed after repeat testing (Evert, Boucher *et al*. 2014).

1.8. Prevention of T2DM

It has been demonstrated through Random Clinical Trials (RCTs) that adapting to good lifestyle habits and through medical intervention the risk of procuring diabetes can be prevented or delayed. A controlled clinical trial study showed that moderate physical activity and controlled diet designed to reduce 5-7% of body weight reduces the diabetes risk by marked 29-58% (Ramachandran, Snehalatha *et al*. 2006)**.** Lifestyle interventions conclude safe, cost effective measures that ensure improved quality of life (QOL) across all ages, races, genders and ethnic groups free of degree of hyperglycaemia and obesity. The risk is reduced by 26-31% by the use of Metformin (Knowler, Barrett-Connor *et al*. 2002)**.** Thiazolidinediones diminishes prospects of T2DM by 55-72% (Investigators 2006) and α-glucosidase inhibitor (AGIs) lowers the possibility by 25-41% (Kawamori, Tajima *et al*. 2009)**.** Investigation of RCT participants showed that beneficial effects healthy lifestyle

choices and interventions may make a positive impact over time (Li, Zhang *et al*. 2008). Efforts on promoting healthy lifestyle changes, primary care and awareness are being sponsored by health ministries in community settings. No authentic medication has been approved by US FDA for prevention of diabetes mellitus.

1.9. Management of T2DM

Multiple Pathophysiological disruptions further complicates the management of T2DM (DeFronzo, Eldor *et al*. 2013) and of management of diabetes i.e. Age, Bodyweight, Complications, Duration, Etiology, Expense and Education (Pozzilli, David Leslie *et al*. 2010)**.** Through glycaemic control T2DM related microvascular complexities could be prevented (Brownlee 2005), while macrovascular complexities could be prevented by reparation of primary cardiovascular risk associates that compose the metabolic insulin resistance syndrome (DeFronzo 2010) (TABLE 1).

Table 1: Intervention to Delay or Prevent T2DM Development in People with Impaired Glucose Tolerance (Meo, Zia *et al*. 2016)

Intervention	Risk Reduction (%)
Lifestyle (body weight reduction 5-7%)	**29-58**
Metformin	**26-31**
Lifestyle and Metformin	**28**
Acarbose (α-glucosidase inhibitor)	**25**
Voglibose (α-glucosidase inhibitor)	**41**
Troglitazone	**55**
Rosiglitazone	**60**
Pioglitazone	**72**

Ideally, the levels of HbA1c should be reduced to the brink of normalcy without causing hypoglycaemia which is a great concern (Raz, Riddle *et al*. 2013)**.** T2DM patients with constant HbA1c levels <6.5% are not at a risk of developing retinopathy (Nakagami, Kawahara *et al*. 1997)**.** The basic cornerstone of all intervention programs (Tuomilehto, Lindström *et al*. 2001) should be lifestyle modification as sedate lifestyle and obesity are states of insulin-resistance linked with lipotoxicity (tissue fat overload) (DeFronzo, Eldor *et al*. 2013). Even though with

initial weight loss many of the patients gain lost weight over a period of 1-2 yr. (Ali, Echouffo-Tcheugui *et al*. 2012). Even after successfully losing weight the close to 50% of obese prediabetic individuals still progress to turn to diabetics (Knowler, Barrett-Connor *et al*. 2002). Hence, most of the experts of obesity management recommend complementary (anti-obesity) medications to help maintain and lose weight. Fat mobilization from the β-cells, muscles and liver reforms muscle and hepatic sensitivity to insulin and the function of β-cells (DeFronzo 2010).

1.9.1. Anti-diabetic Treatment

Long term glycaemic control could only be achieved through effective antidiabetic medications that can turnabout the Pathophysiological defects associated with T2DM. A combination therapy is preferred as no single medicine can reverse the spectrum of abnormalities related with T2DM (Abdul-Ghani, Migahid *et al*. 2017). Combination therapy is widely accepted by practitioners (DeFronzo, Lewin *et al*. 2015). Normal HbA1c levels in the period of diagnosis of T2DM can help attain durable glycaemic control (Weng, Li *et al*. 2008). For Prediabetics, the onset of IGT could be effectively delayed or prevented by the combination therapy of metformin (Knowler, Barrett-Connor *et al*. 2002), GLP1 receptor agonists (Astrup, Carraro *et al*. 2012), thiazolidinediones (Xiang, Peters *et al*. 2006), and AGIs (Chiasson, Josse *et al*. 2002)**.**

1.9.1.1. Metformin

Metformin stands out as the most commonly prescribed drug for the management of T2DM worldwide. It acts by inhibiting hepatic glucose production which ultimately leads to reduced fasting plasma glucose levels and HbA1c levels (Cusi, Consoli *et al*. 1996). Metformin does not affect β-cell functioning (Turner, Cull

et al. 1999) and in case of inadequate weight loss, the muscle insulin sensitivity does not improve (Cusi, Consoli *et al*. 1996) so after an initial decline in the levels of HbA1c it increases progressively (Kahn, Haffner *et al*. 2006)**.** It is rather unclear how metformin acts in suppression of hepatic glucose production but it involves the stimulation of AMPK (AMP-activated protein kinase), restriction of mitochondrial complex I, mitochondrial glycerophosphate dehydrogenase (Madiraju, Erion *et al*. 2014), inactivation of gluconeogenic and/or glycolytic enzymes (Ferrannini 2014). According to a study done in United States of America and United Kingdom of about 344 obese subjects with diabetes showed that metformin reduced the risk of cardiovascular complications significantly (Group 1998)**.**

1.9.1.2. Insulin Secretion Enhancing Drugs

Sulfonylureas are a class of drugs that reinforces secretion of insulin which results in hyperinsulinaemia that can overcome insulin resistance consequently leading to decreased HbA1c levels and fasting plasma glucose levels. Since, β-cell functioning is not shielded by sulfonylureas, so after an initial decrease the HbA1c levels increase gradually (Madiraju, Erion *et al*. 2014) and might even result in hastening the β-cell functions failure (Maedler, Carr *et al*. 2005)**.** This drug is linked with weight gain and is commonly known to cause hypoglycaemia and some studies have found that it may raise the risk of cardiovascular complications (Kahn, Haffner *et al*. 2006). Compared to metformin the stepwise increase in sulfonylurea is linked with progressive failure of function of β-cells and an increase in HbA1c levels (Yki-Järvinen 2004). However, metformin and sulfonylurea stand out as the most prescribed oral antidiabetic drugs worldwide due to their inexpensiveness and availability.

Insulin secretagogues such as Meglitinides (nateglinide and repaglinide) act for a short while and have to be administered before every meal as required. These drugs do not promise the prevention of gradual decline of β-cells and rise in levels of HbA1c that is classic indication of T2DM. They are related with less hypoglycaemia than sulfonylurea (Yki-Järvinen 2004).

1.9.1.3. Insulin Sensitizing Drugs

The only renowned insulin sensitizers are Thiazolidinediones (rosiglitazone and pioglitazone) (Eldor, DeFronzo *et al*. 2013). They act by augmenting insulin sensitivity in cardiac muscles and skeletal, the adipocytes and the liver (Miyazaki, He *et al*. 2003) and have powerful action on β-cells to enhance and preserve insulin secretion (Gastaldelli, Ferrannini *et al*. 2007)**.** Several mechanisms negotiate the effects of insulin sensitization: rise in stimulation of insulin; triggering of Peroxisomes Proliferator-Activated Receptor-γ (PPARγ); increased fat oxidation by activation of PPARγ co activator 1 (PGC1); incitement of various intracellular steps implicated in glucose metabolism (glycogen synthase, pyruvate dehydrogenase and GLUT4); redistribution of fat to subcutaneous from visceral stores; subcutaneous Adipocytes propagation and stimulation lipogenesis causing genes; decline in plasma levels of FFAs; rise in the levels of adiponectin and a decline in cytokines level (Eldor, DeFronzo *et al*. 2013)**.** Thiazolidinediones are considered to have a durable double action on β-cell stimulatory effect and insulin sensitizing and their potential to markedly lessen HbA1c (Dormandy, Charbonnel *et al*. 2005). Pioglitazone cordially affected the MACE bounds i.e. myocardial infarction, stroke and cardiovascular death) and positively influences factors of insulin resistance syndrome (Eldor, DeFronzo *et al*. 2013).

1.9.1.4. Modulators of GLP1

Serious GLP1 resistance in β-cells is associated with T2DM (Kjems, Holst *et al*. 2003). The half-life of endogenously secreted GLP1 could be drawn-out by the use of Dipeptidyl peptidase 4 (DPP4) inhibitors as (saxagliptin, alogliptan, linagliptin, sitagliptin and vildagliptin). The action of DPP4 inhibitors in declining HbA1c levels and to enhance insulin sensitivity is modest at its best as it does not increase plasma GLP1 levels but just prolongs their half-life (Deacon 2011). Improvement of glycaemic control is their major effect and is negotiated by lowering the basic hepatic glucose production (Balas, Baig *et al*. 2007). DPP4 inhibitors are considered to have great safety profile (Drucker 2013)**.** The agonists of GLP1 receptors such as liraglutide, dulaglutide, exenatide and lixisenatide distinctly strengthen the secretion of insulin and restrict glucagon secretion (Cervera, Wajcberg *et al*. 2008)**.** The rise in insulin levels of plasma and the decline in the glucagon levels efficaciously repressed hepatic glucose production (Cervera, Wajcberg *et al*. 2008) are responsible in a strong reduction in HbA1c levels for up to 3 years (Bunck, Cornér *et al*. 2011). GLP1 receptor agonists effectively enhance weight loss that promotes improved sensitivity to insulin, corrects debilitated endothelial functioning, positively impacts the plasma lipid profile, and normalizes blood pressure, reduction in the levels of C-reactive proteins and dilatory gastric emptying (Schwartz and Kohl 2010). A combined therapy with GLP1 receptor agonists and basic insulin therapy can together work to lower HbA1c while discouraging weight gain linked with insulin therapy, all the while controlling the risk of hypoglycaemia (Eng, Kramer *et al*. 2014)**.**

1.9.1.5. Insulin Reinforcement

T2DM patients are put on an insulin reinforcement program where conventional oral or injectable antidiabetic drugs that are unable to control the HbA1c

levels. Often abundant doses (>80-100 units/day) of insulin are required (Holman, Farmer *et al*. 2009). Though, insulin dose could be substantially reduced by the administration of thiazolidinediones and metformin to enhance glycaemic control. GLP1receptor agonists and insulin therapy can bring about a robust reduction in HbA1c levels, simultaneously promoting decrease in weight and insulin dose (Gough, Bode *et al*. 2014)**.** Combination therapy of SGLT2 inhibitors together with insulin can pose effective in reducing HbA1c, insulin dose reduction, lower risk of hypoglycaemia and promotion of weight loss. Intensive insulin therapy as a new age treatment of T2DM has shown positive results of maintaining glycaemic control (HbA1c approx. 6.0%) for longer periods and also reversed the metabolic decompensation as lipotoxicity and glucotoxicity(Weng, Li *et al*. 2008)**.**

1.9.1.6. Potential Adverse Effect of All Anti-diabetic Agents - Hypoglycaemia

Insulin secretagogues such as insulin and sulfonylureas significantly increase the risk of causing hypoglycaemia in T2DM patients (Anderson, Powell *et al*. 2014)**.** According to American Association of Diabetes (ADA) plasma glucose levels of <70mg per dl are characterized as hypoglycaemia. Hypoglycaemia has been closely linked with ventricular arrhythmias, myocardial infarction, weight gain and stroke (Anderson, Powell *et al*. 2014). Use of insulin and sulfonylureas, strenuous exercise, other drug interaction, skipping meals, hepatic or renal disease, consumption of alcohol and advanced age have been linked as risk factors of hypoglycaemia (Group 2008)**.**

1.10. Objective of the Study

To investigate the presence of single nucleotide polymorphisms of VEGF-A (rs699947) and VEGF-A (rs2146323) in association with T2DM in Pakistani patients as a causative agent and risk factor for promoting DR.

Chapter 2

Literature Review

2.1. Type 2 Diabetes Mellitus (T2DM)

Mention of diabetes dates back to antiquity, where manuscripts have been found (as old as 1500 BC) mentioning 'passing of the urine' in 'great quantity' (Zajac, Shrestha *et al*. 2010). Type 2 diabetes is considered as a Global Epidemic by the World Health Organization. It is also known as adult-onset or insulin-dependent diabetes and results from the body's ineffective use of insulin. It is a long-term metabolic disorder, with lack of physical activity and excess body weight as its main causative factors. Genetics too play a role and some people are more at risk than others. Majority of people around the world suffering from diabetes are Type 2 cases. Their symptoms are often less marked in comparison to Type 1; resultantly complications may already have arisen when diagnosis is made. Type 2 was considered an adult onset disease but recently it has been observed in children as well. In 2015 there were approximately 392 million people diagnosed with the disease compared to around 30 million in 1985 (Vos, Abajobir *et al*.). Type 2 diabetes is associated with a shorter life duration and is diagnosed mostly in middle or older ages. Symptoms include frequent urination, increased thirst, and unexplained weight loss. Symptoms may also include increased hunger, feeling tired, and sores that do not heal. Symptoms often come on slowly. High blood sugar also has long term complications that include heart disease, strokes, diabetic retinopathy, kidney failure, and poor blood flow in the limbs which may lead to amputations. Therefore, change

of lifestyle and medication is a must to offset the effects of diabetes, which is aptly dubbed as a global epidemic.

2.2. Diabetic Retinopathy (DR): Microvascular Complication of T2DM

Diabetic Retinopathy is a renowned repercussion of persistent and subnormal control over diabetes mellitus (DM). It is one of the leading causes of vision loss and blindness worldwide with some 150 million people affected globally. According to UN World Health Organization (WHO) the numbers of affected people will double the current rate in 2025 (King, Aubert *et al.* 1998).

Prevalence of any type of DR in patients of DM is close to 24% (Gupta, Mansoor *et al.* 2013)**.** One of the major blindness causing factors of DR is Diabetic macular edema (DME). It has been cited as the results of a Wisconsin Epidemiologic Study that patients with 15 year or more duration of DM have a prevalence of DR approximately 25% in patients of T2DM and 20% in patients of T1DM that are on treatment (Klein, Klein *et al*. 1984). Patients with advanced level of DME can still maintain their good sight with appropriate management of the disease. Therefore, timely management of DME and treatment are the first priority of ophthalmologists in preventing total blindness of the DM patients.

Diagnosis of Macular edema is clinically done by a biomicroscopic examination by recording the hard exudates in the retina or its thickening. A laser treatment has been recommended for the type of DME known as “Clinically Significant Macular Edema” (CSME) under the published research of The Early

Treatment Diabetic Retinopathy (ETDRS) (Group 1987). Angiographic documentation suggests that maculopathy arising from the diabetes is characterized either as diffused or focal, and non-ischemic or ischemic. Diabetic maculopathy caused by leakage in the capillary bedding is known as focal. And the one caused by percolation of microaneurysms is characterized as focal. Due to blood deprivation to the ischemic zones in retina, the prognosis of vision is poor.

Although diabetic retinopathy is a disease characterized by the vascular and ischemic changes in the retina but in the more advanced stages, similar changes can be found in other parts of the eye. Extensive ischemia of the eye retina is caused by neovascularization of the retina (NBE), the angle (NBA), the disk (NVD) and the iris (NVI). Neovascularization of the angle causes neovascular glaucoma (the derivation of the closure of the angle). For many years, as a treatment for these conditions the retrogression of the anomalous new vessels on the angle and iris were brought about treating them with panretinal photocoagulation (PRP) of the retina (Gardner, Tai *et al*. 2012). The neoglaucoma that causes synechial closure of the angle is known to be progressive in its stage. Such cases are facilitated with aqueous drainage of the retina by the help of placement of Ahmed glaucoma valve or a surgical shunt as the likes of Baerveldt tube shunt. Recently, for the treatment of rubeosis, an anti-VEGF drug is being investigated. Over a period of nine months, ranibizumab injections are employed under the (rubeosis anti-VEGF) clinical trial for Ischemic Central Retinal Vein Occlusion (CRVO) to detect if such a treatment can overcome neovascular glaucoma prognosis in the eyes with.

2.2.1. Medical Care of Diabetic Retinopathy

One of the two most cardinal methods to control the threat of blindness DME is comprehensive glycemic check. A research done under UK prospective Diabetes study (DeFronzo and Abdul-Ghani 2011) and diabetes control and complications trial (Bloomgarden 2003) showed its findings that restricting blood glucose levels around normal can regress the risk of DME development by 23%.

The other major route of action is laser photocoagulation to control loss of vision from DME. ETDRS and clinical trials done under the diabetic retinopathy study (DRS) program have displayed that the risk of blindness is lowered by 90% by a timely IPL intervention (Ferrannini, Gastaldelli *et al*. 2011). For a diabetic retinopathy, that is proliferative in nature PRP laser is the choice of treatment as shown by various clinical trials. Sadly, PRP laser scars and creates loss of function in the area of retina treated by it.

In the DME patients' eyes the decrease of angiogenesis could be brought about by inhibiting the expressions of VEGF through corticosteroids (Nathan, Davidson *et al*. 2007). Proinflammatory cytokine known as platelets-derived growth factor (PDGF) is responsible for triggering the expression of VEGF gene. For retinal disorders intravitreal injections of triamcinolone acetonide (IVTA) are widely used. In advanced cases of DR visual outcomes are classically poor accompanied with complications such as deep-rooted vitreous hemorrhage (VH), fibrovascular proliferation and tractional retinal detachments (TRD). In such cases, the procedure of choice is surgery (Zinman, Harris *et al*. 2010). Patients with diabetes and concomitant hypertension are at a greater risk of cultivating DR and DME. To top it all, the progression of DR is more rapid in such patients (Purcell, Sumithran *et al*. 2014). The

regulation of blood pressure and maintenance of water balance is regulated by rennin-aldosterone system (RAAS). This system is in directly connected to the pathological microvascular changes involved in DR (Tuomilehto, Lindström *et al*. 2001). Angiotensin-II of the RAAS is a primary bioactive product that acts as a potent vasoconstrictor of arterioles and also as an endocrine, endocrine and paracrine hormone. It has been shown by *in vitro* studies that the secretion of VEGF is directly stimulated by angiotensin-II in cardiac endothelial cells (Bloomgarden 2003) and in culture smooth muscle cell (Inzucchi, Bergenstal *et al*. 2012). Accordingly, the narrowing the retinal microvasculature could issue local elevation in production of VEGF within the retina, which is followed by angiogenesis (Inzucchi, Bergenstal *et al*. 2012). The complete pathway linking increased production of VEGF to angiotensin-II is yet to be decoded but hypoxia-inducible factor (HIF-1α) has been shown to implicate at the intracellular signaling pathway level.

2.3. Historical Background of VEGF Molecule

In 1948 Isaac Michelson reported “Factor X” released by the ischemic eyetissue which was involved in the expansion of pathological angiogenesis. He termed the production and release of this diffusible angiogenic molecule as a key event in developing retinopathy (Federation 2013). In 1971, Folkman’s idea of regressing angiogenesis for the treatment of cancer led to the use of anti-angiogenic drugs (Hu 2011). Ferrara and colleagues in 1989, reported a particle in bovine pituitary follical cells that were responsible for the synthesis for the propagation of endothelial cells and named it as vascular endothelial growth factor (VEGF) (Ley, Hamdy *et al*. 2014). Two independent research studies in 1992, reported that expression of VEGF could be up-regulated by hypoxia (Cappuccio, D'Elia *et al*.

2010). Hence, the integral role of VEGF in propagating ocular neovascularization was backed up by such clinical studies. It was proved by vitreous samples with active propagative retinopathies linked with venous occlusive disorders and with diabetes had raised levels of VEGF (Barnett, Eff *et al*. 1981).

2.4. The VEGF Molecule

A subfamily of growth factors that behave as signaling proteins for new blood vessel growth from previously existing vasculature and vasculogenesis otherwise known as angiogenesis the de novo production of the circulatory system and the embryonic stage, possesses a member known as Vascular endothelial growth factor (VEGF). Primarily, the discharge of VEGF is from the pigmented epithelial cells of the retina, astrocytes, Müller cells, glial cells, pericytes and endothelial cells. VEGF is a group of various members inclusive of placental growth factor (PGF), VEGF-A, VEGF-C and VEGF-D. VEGF-A is a 36KDa of glycosylated protein which is derived from alternate splicing of mRNA of a single VEGF-G (Wang, McPherson *et al*. 2011). The variance of VEGF-A expression depends on the presence of number of amino acids it is then expressed as 5 isoforms or mRNA splice variants - 121, 145 and 206. The heparin binding ability is magnifying in larger isoforms (isoforms 189 and 206) which indicates their potential to bind two basement membranes and cell surfaces. Unfortunately, their diffusibility of larger isoforms is either nil or limited. On the other hand, the smaller isoforms are promptly disseminated but restricted heparin binding potential persists. Isoform 165 has median potential of heparin binding and diffusiblity. VEGF-165 is the major secreted isoform which is crucial for the growth and abnormal angiogenesis (Wang, Bao *et al*. 2013). The other

constituents of the VEGF family may play a more pronounced role in cancers and tumors than in DR (Li, Shin *et al*. 2009).

2.5. Production of VEGF

VEGF is produced in response to hypoxia and ischemia (Figure 3). Hypoxia in the tissue is responsible for triggering the synthesis of a DNA-binding protein known as HIF-1 (hypoxia-inducible factor 1). It is an elementary helixx-loop-helixx, heterodimeric protein with two elemental sub-units: the one regulated by a growth factor called HIF-1 alpha subunit and the other is integrally expressed HIF-1 beta subunit (Ding, Song *et al*. 2009). HIF-1 is not only crucial for synthesis of VEGF but it also upregulates the production of a gene called human erythropoietin in the cells that are undergoing hypoxia that is another variation of hypoxia of the tissue (Wang, Larson *et al*. 2011)**.** The binding of HIF-1factor to VEGF gene triggers the transcription of the gene. This leads to a hyper-production and aggregation of the VEGF mRNA due to hiked transcription of mRNA and depressed mRNA degradation (Esteve, Ricart *et al*. 2011)**,** as a result the VEGF molecules are amassed intracellular spaces. VEGF is transported from endoplasmic reticulum to Golgi bodies via a chaperon protein known as ORP 15 (oxygen regulated protein). The ORP15 expression is also hypoxia induced (Hu, Manson *et al*. 2001).

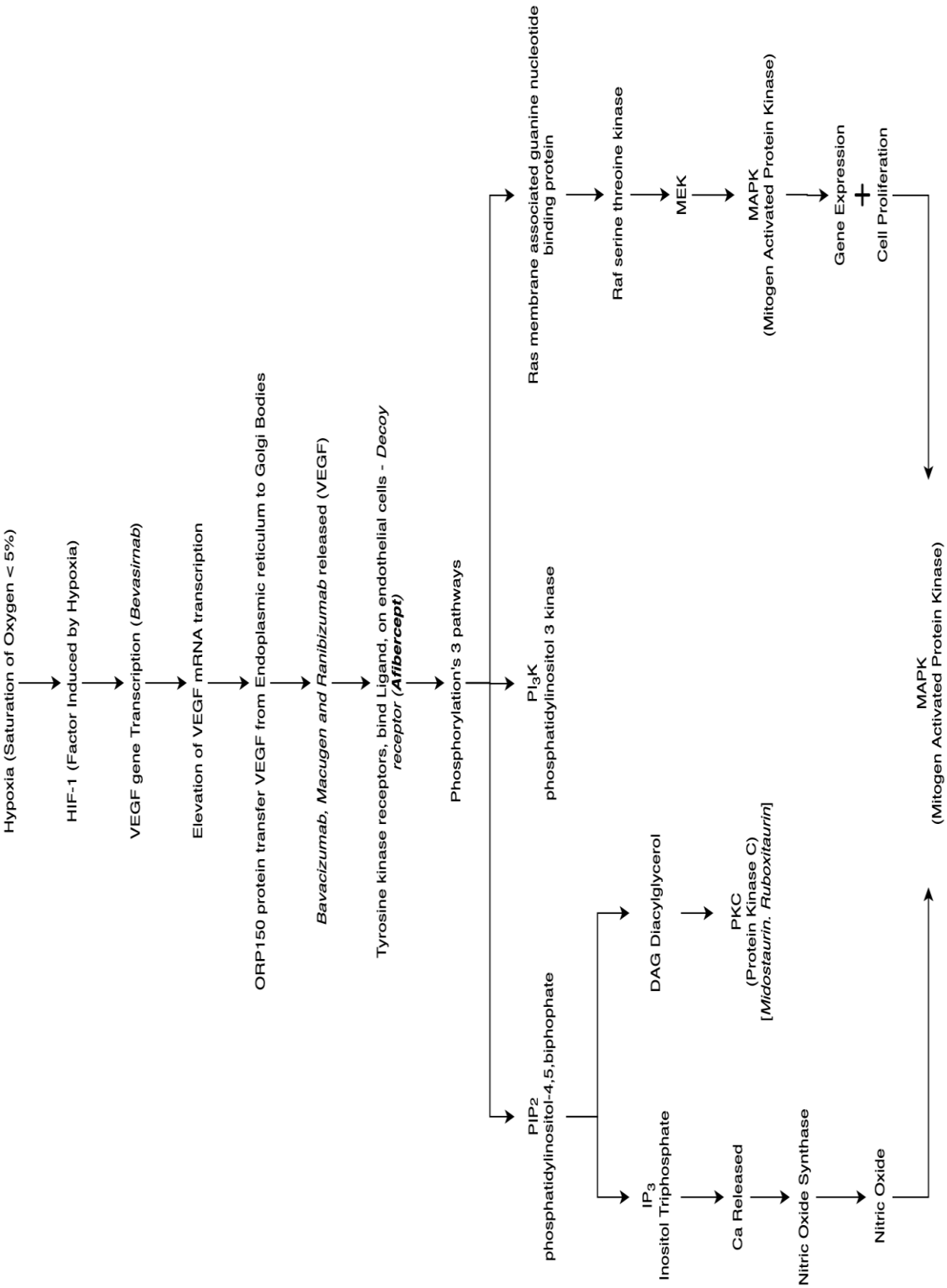


Figure 4: A VEGF cascade flowchart demonstrating the molecule chain events that occur after hypoxia in the retinal tissue resulting in angiogenesis (Gupta, Mansoor *et al*. 2013).

2.6. VEGF-A Isoforms – Generation by Alternative Splicing

Vascular supply greatly relies on rigid regulation between elements that are responsible for anti-angiogenic and pro-angiogenic development of vessels through a mechanism that is highly sensitive to subtle changes in nutrient levels and oxygen. The human VEGF-A gene is situated on chromosome 6p21.1 (Johnson, Lin *et al*. 2017) with a coding region that is approximately 14 kilobases long based on seven introns and eight exons. The pre-mRNA when alternatively spliced, the introns are selectively removed and specific exons are combined together to produce defined VEGF-A isoforms (Peach, Mignone *et al*. 2018) (Figure 1). Alternative splicing aids in expanding the repository of possible isoforms of VEGF-A from a single gene (Peach, Mignone *et al*. 2018). The isoforms are distinguished by their length and are designated by $VEGF_{xxx}$ where xxx denote the number of amino acids in the eventual protein sequence. Up till now, 16 various isoforms of VEGF-A have been reported most frequently originating from six transcripts: $VEGF_{111}$, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Carter, Gammons *et al*. 2015). The first isoform to be characterized was $VEGF_{165}$ and to date remains to be the most investigated regarding its expression, signaling, function and any pathological roles (Kazerounian and Lawler 2018). $VEGF_{165}$ is a powerful instigator of angiogenesis and is treated as prototypical angiogenesis inducing VEGF-A isoform. Modified expressions of VEGF-A isoforms have been well studied in tissues during certain pathological and physiological conditions (Barratt, Blythe *et al*. 2017). VEGF-A splicing is regulated by spliceosomes in the nucleus and small nuclear ribonuclear proteins (snRNPs) along with accessory proteins SF1 and U2AF; a set of RNA binding proteins majorly the serine/arginine (SR) proteins like SRSF1, SRSF2, SRSF5 and SRSF6 (Barratt, Blythe *et al*. 2017). Phosphorylation of SR proteins is brought about in the cytoplasm at the

various proline/serine and serine/arginine repeats to bring around their translocation to nucleus and allow a certain degree of spatial splicing regulation. In the nucleus, SR proteins affix to regulatory sites in pre-mRNA of VEGF-A which instigates removal of the exons (Batson, Toop *et al*. 2017). Any changes in SRPK1 expression have been highlighted in a series of malignancies. So the SRPK1 inhibitors were designed to direct corrective action towards any aberration in angiogenesis through changing splicing of endogenously found VEGF-A isoforms (Batson, Toop *et al*. 2017).

Different exons are responsible for conferring various properties to the isoforms (Figure 5). Constitutive exons present in all the isoforms of VEGF-A are exons 1-5. A signal sequence is encoded by them (exons ½) eventually cleaved in a bid to form processed VEGF, they also include a glycosylation site (Asp74), residues that bind VEGFR1 and VEGFR2 and a possible plasmin cleavage site (Arg110 and Ala111) (Stevens and Oltean 2019). One of the major splice sites of VEGF gene is located around on exons 6 and 7. The residues in exon 6a and 7 are responsible for forming links with electronegatively associated heparin sulphate situated in the extracellular matrix which has a major impact on the bioavailability of isoforms (Chiodelli, Bugatti *et al*. 2015). The shorter length isoforms such as the $VEGF_{111}$ and $VEGF_{121}$ are freely diffusible as they are not tethered in the Extracellular Matrix (ECM) because they lack exons 6 and 7 (Houck, Leung *et al*. 1992). In comparison, the isoforms of longer sequences such as the $VEGF_{145}$, $VEGF_{189}$ and $VEGF_{206}$ that possess exons 6a and 7 can attach with high affinity glycoproteins as heparin sulphate (Houck, Ferrara *et al*. 1991) (Figure 5).

Another major site of splicing is hosted by the option of differential 3’ splice acceptor sites in exon 8. $VEGF_{xxx}a$ and $VEGF_{xxx}b$ isoform differ on the bases of

sequence. Their different sequences of six amino acids at their C-termini; the isoform $VEGF_{xxx}a$ terminates in CDKPRR, and $VEGF_{xxx}b$ ends in SLTRKD (Ladomery, Harper *et al*. 2007). $VEGF_{xxx}a$ isoforms are considered to be pro-angiogenic as they are found to be prime instigators of cell proliferation, migration, angiogenesis, survival and vascular permeability (Olsson, Dimberg *et al*. 2006). Whereas, $VEGF_{xxx}b$ have been found to have anti-angiogenic properties (Woolard, Wang *et al*. 2004).

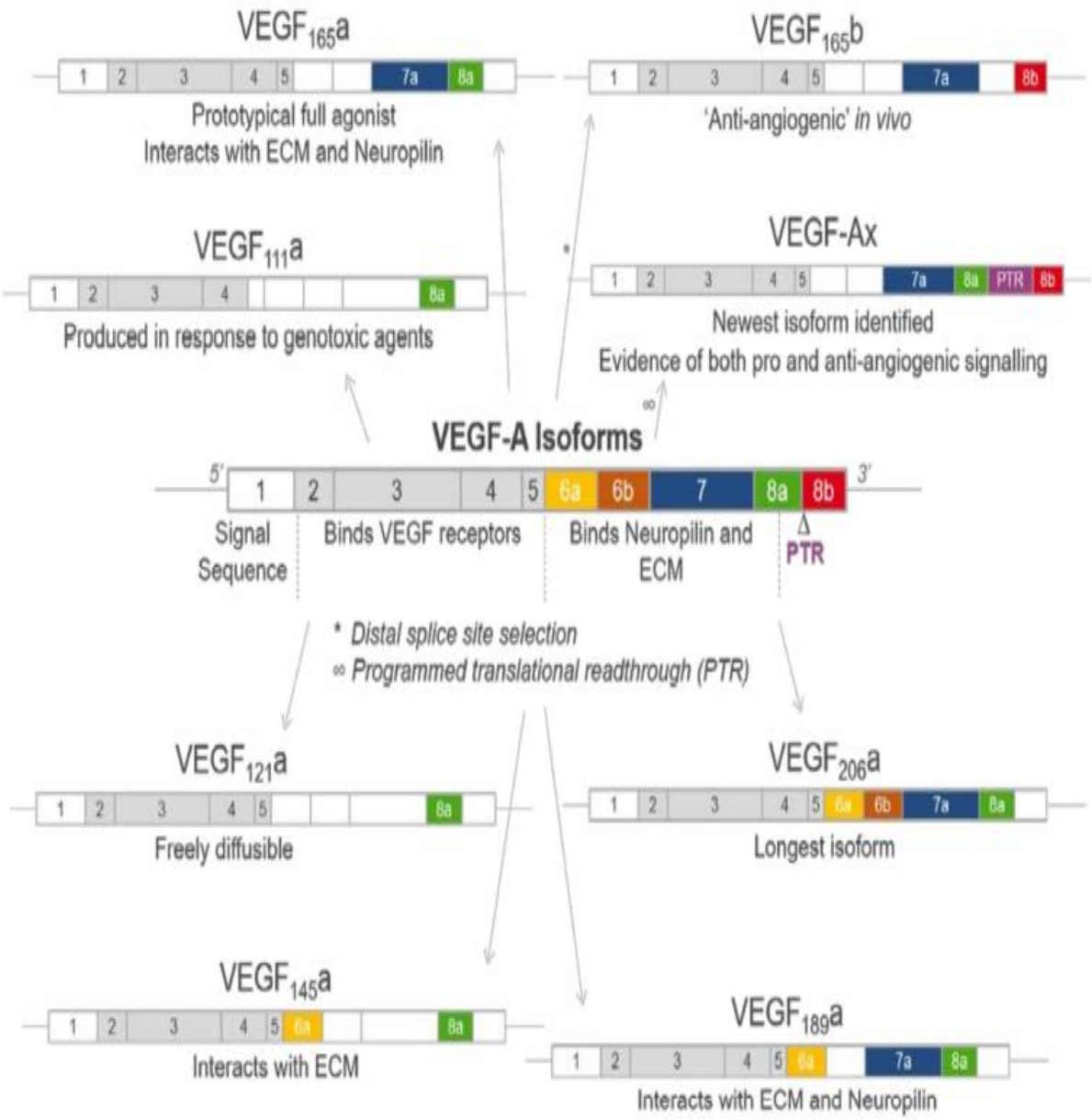


Figure 5: Schematic Illustration of the Structure of VEGF-A Isoforms | The eight exons gene VEGF-A is alternatively spliced if required to generate various VEGF-A isoforms. The isoforms differ from each other on the basis of length are denoted by $VEGF_{xxx}$ where xxx stands for the number of amino acids present in the sequence. Every exon possesses residues which imparts specific properties to the resultant isoform, including the extracellular matrix (ECM), VEGFR2 and Neuropilin (NRP) binding. Exon 8 is the typical primary site of alternative splicing which generates prototypical $VEGF_{xxx}$a isoform after proximal splicing and distal splicing results in $VEGF_{xxx}$b isoform with exon 8b. Through the use of a non-canonical stop codon, the post translational read-through (PTR) generates VEGF-Ax isoform that has 22 amino acid extension within its C-terminal domain (Peach, Mignone *et al*. 2018).

2.7. Mode of Action of VEGF

The binding of all VEGF family units to the receptors of tyrosine kinase receptors over the surface of the endothelial cell that stimulates a cellular response, causing a dimerization of the VEGF molecule and its activation through transphosphorylation. Two kinds of receptors are possessed by VEGF-A: VEGF receptor 1 (Flt-1 or VEGFR-1) and VEGFR-2 (KDR or VEGFR-2). VEGFR-1 is encoded by a gene called Flt-1 gene in humans (Hu, Manson *et al*. 2001)) and the KDR (kinase insert domain receptor, which is a kind of type III tyrosine kinase receptor) or VEGFR-2 which is encoded by a gene KDR (Schellenberg, Dryden *et al*. 2013)**.** The primary receptor responsible for navigating cellular responses to VEGF-A is VEGFR-2. The receptors of VEGF are composed of three parts: a tyrosine kinase domain contained in an intracellular portion, seven identical immunoglobulin-like domains and a hydrophobic spanning region with a single transmembrane (DeFronzo 2010). A cellular signal is transduced through intracellular portion of VEGF receptor by the phosphorylation of the tyrosine amino acid residues as a result of the binding of VEGF molecule to the extracellular region of VEGFR (DeFronzo 2010). A cascade of intracellular signaling pathways is triggered by this transduction. A protein kinase C (PKC) pathway is caused by VEGF within the cells (Hemminki, Li *et al*. 2010). Angiogenesis is also brought about by other pathways through the formation of nitric oxide and inducement of cellular propagation.

2.8. Angiogenesis in DR by VEGF

A major determinant in the induction of proliferative DME and DR is VEGF gene (Groop, Forsblom *et al*. 1996). It alters the permeability of retinal capillaries by

up regulating the phosphorylation of the proteins engaged with tight junctions as the like of zonula occcludens (Lyssenko, Lupi *et al*. 2007)**.** Induction of VEGF stimulates MAP (mitogen-activated proteins) that results in proliferation of endothelial cells. This particular cascade concurs with the stimulation of phosphatidylinositol 3-kinase (PI3)/Akt pathway after the induction of VEGFR-2 (Wang, Larson *et al*. 2011). Endothelial cells are triggered by VEGFA to secrete urokinase-type plasminogen activator and Matrix Metalloproteinases (MMPs) which in turn is responsible for degeneration of basement membranes and creating the possibility for the cells to migrate. Such activated endothelial cells manifest the integrins as the likes of αvβ5 and αvβ5, which facilitates the movement of the cells through the disintegrated matrix (Grant, Thorleifsson *et al*. 2006)**.** After the endothelial cells proliferate and migrate the basement membranes are synthesized for the freshly manufactured capillaries.

2.9. VEGF as a Therapeutic Objective

Several mechanisms of VEGF have been studied to down regulate its effects that cause the vessel leakage and neovascularization by countering its effects. Figure 3 demonstrates the sites where anomalous neovascularization is targeted by the drugs.

2.9.1. Anti-VEGF Antibodies

Two drugs are available under this class commercially. The drug Bevacizumab is a monoclonal humanized antibody of full-length which acts against all the isoforms of the gene VEGF-A and can prevent receptor binding on the VEGF molecule. USFDA has approved it for commercial intravenous drug treatment for colon cancer that is metastatic. Though USFDA has not approved bevacizumab for intravitreal treatment for the diseases of the eye, it has been administered off label and

has been proved to be very effectual in hindering neo-vascularization linked with proliferative illnesses of the retina as CNV, macular edema, neovascular glaucoma, proliferative diabetic retinopathy and retinal vein occlusions (Travers, Mackay *et al*. 2013).

The other known drug is Ranibizumab was formulated as slight antibody fragment for an effective intraocular penetration inside the retina. It was endorsed in 2006 by USFDA for the treatment of neovascular (wet) age-related macular degeneration (AMD). Initial studies have shown promising results with intravitreal ranibizumab showed a decrease in average thickness of the retina and a comparatively improved vision in DME patients (Saxena, Voight *et al*. 2007).

2.9.2. Intracellular Transcription Inhibitors

The first known drug designed to silence the small interfering RNA (siRNA) to hamper the synthesis of VEGF by silencing the genes that produce it, is Bevasiranib. The RACE trials that were a part of the experimental trial phase II of investigation studied 3 dose levels of bevasiranib and reported thinness in macular width between 8 and 12 weeks. It also showed that the higher doses of the drug caused more shrinkage of thickness than the lowest drug dose (Ferrannini and Mari 2014).

2.9.3. Extracellular VEGF Inhibitors

For neovascular AMD treatment the earliest VEGF inhibitor drug approved by the USFDA is a RNA aptamer by the name of Pegaptanib sodium. It fastens to VEGF isoform 165 and blocks its action (Kahn, Cooper *et al*. 2014). A recent study of randomized controlled trial in phase II/III showed the results that with Pegaptanib

therapy the visual outcomes of subjects with DME significantly improved for as long as 2 years (Muller, Elahi *et al*. 1996).

2.9.4. VEGF Receptor Expression Inhibitors

Aflibercept (VEGF-Trap), is a drug formulated as a total human recombinant fusion protein that has the ability to bind with all the types of VEGF-A and PGF (Placental Growth Factor) (Madsbad 2014). The companies are designing the drug to be used as a treatment for ocular use. The trial under the DA VINCI research study gave encouraging results compared to conventional laser photocoagulation in DME cases. A statistically marked improvement was observed in visual sharpness in the patients treated during the VEGF Trap-Eye Trial at the major endpoint observation of 24 weeks and also through the follow up study of 52 weeks. In addition, the reduction in the retinal thickness and optical coherence tomography was also observed. Currently, a diffused clinical trial has entered in phase III is investigating the efficiency of VEGF Trap-Eye medication for the treatment for diabetic macular edema (DME) (Do, Nguyen *et al*. 2009; Do, Schmidt-Erfurth *et al*. 2011). It has been noted that PKC412 showed promising inhibition of neovascularization induced by ischemia in an animal model (Lopez Galvez 2009). However, primal pharmacodynamic research has displayed conflicting results, especially highlighting its absence of specificity. The basic toxicities caused by a dose of PKC412 are fatigue and vomiting/nausea. It was found that the optimum tolerated dose of PKC412 is 150mg/d to be administered to chronic cases (Propper, McDonald *et al*. 2001). The optimum dose is even hepatotoxic, hence, Ruboxistaurin - a more selective drug is opted (Campochiaro 2004).

2.9.5. Prohibition of Intracellular Signaling Pathway Activating VEGF

One of the potent prohibitors of VEGF receptor signaling is Midostaurin that prevents the neovascularization in the retina (Wang, Yin *et al*. 2008). It is also a powerful inhibitor of the various isoforms of VEGF-R2, KIT tyrosine kinase, protein kinase C (PKC), Flt-3 tyrosine kinases and PDFGR (platelet-derived growth factor receptor) (Fabbro, Ruetz *et al*. 2000).

2.9.6. Inhibitors of Protein Kinase C (PKC) Beta

An orally administered selective inhibitor of PKC beta isoform is Ruboxistaurin. It is a drug still under investigation for treating severe to moderate non-proliferative DR. The (PKC-DRS) PKC-Diabetic Retinopathy Research Study was designed to investigate the action of ruboxistaurin on loss of vision of non-proliferative subjects of DR (ranging from moderate to severe). It displayed remarkable decline in the chance of occurrence of moderate visual hampering (doubling of the visual angle) and sustained moderate loss of vision of around 6 months. The study PKC-DME reported slowed progression of DME with the administration of Ruboxistaurin, although the drug has not been approved yet by the USFDA (Association 2005; Girach, Aiello *et al*. 2006; Group 2006; group 2007).

Chapter 3

Methodology

3.1. Sample Collection and Sample Size

The research was conducted to study the prevalence of *VEGF-A (rs699947)* and *VEGF-A (rs2146323)* in the population of T2DM patients in comparison to normal controls. Total subjects enlisted for this study were 186 and the samples were collected from Military Hospital, Rawalpindi, PIMS Hospital, Islamabad, Amna Hospital Sialkot and Sialkot Medical Complex, Sialkot over the period of 5 months. The sample collection was conducted keeping in mind the following criteria:

i. Control Group – Healthy subjects without T2DM (80 samples)
ii. Subjects with Type 2 Diabetes mellitus (106 samples)

3.2. Criteria of Inclusion and Exclusion

Patients of both genders male and female were made a part of this study under both the designated groups of the study. The allotted age range for the subjects was as followed:

i. For Subjects under T2DM – 40 years and above
ii. For Controls – 25 years and above

The subjects diagnosed with chronic and contagious illnesses such as Hepatitis B, C and AIDS were excluded from the study. Only the subjects diagnosed with

T2DM were enrolled in the study. Others with T1DM and gestational diabetes were excluded.

3.3. Collection of Blood Samples

To collect the blood samples under ethical permission a grant of approval was required by the Institutional Review Board (IRB), ASAB. Blood samples from the subjects were collected in a 5ml EDTA (Ethylenediaminetetraacetic) tubes under the care of a phlebotomist. The tubes were then labeled with a certain ID known in the laboratory, name, age of the patient and date of collection of the sample. The consent of all the subjects was taken on a consent form along with their contact details before the blood was drawn. The samples were carefully transported to the laboratory in an ice box and were transferred immediately in a 4ºC refrigerator upon arrival in the laboratory.

3.4. Extraction of DNA from the Blood Samples

Once the samples were brought back to the Immunogenetics laboratory at ASAB, the extraction of DNA was conducted by the Phenol-Chloroform method over a period of two days.

3.4.1. Day 1 of Phenol-Chloroform DNA Extraction

i. Through the pipette, 750 µl of blood samples was measured and transferred to 1.5ml centrifuge tubes (Axygen California, USA), which were autoclaved before use. An equal amount of i.e. 750 µl of Solution A was mixed with the blood in a centrifuge.

ii. The tubes containing the mixture of blood and solution A were inverted 4-6 times and were then incubated for 5-10 minutes at room temperature.

iii. After the incubation period, the tubes containing the solution of blood and solution A were centrifuged for 1 minute at 13,000 rpm in a microcentrifuge.

iv. The supernatant that forms over the mixture was discarded from the centrifuge tubes and the obtained pellet was resuspended in a 400µl of Solution A. The tube containing the pellet and Solution A was centrifuged again at 13,000rpm for 1 minute.

v. The supernatant obtained was cast away and the pellet was re-suspended in 400µl of Solution B, 5µl of Proteinase K and 12µl of 20% SDS and the tubes were then incubated at 37ºC for approx. 24 hrs.

3.4.2. Day 2 of Phenol-Chloroform DNA Extraction

i. The samples incubated a day before were removed from the incubator and were mixed with 500µl of freshly prepared combination of Solution C and D. For 10 minutes the mixture in Eppendorf tubes was centrifuged at 13,000 rpm.

ii. The aqueous phase acquired after the centrifugation was transferred into new Eppendorf tubes and 500µl of Solution D was put in the sample mixtures and were spun again in a centrifuge for 10 minutes at 13,000rpm.

iii. The obtained aqueous phase was shifted to a new tube and 55µl Sodium acetate (pH 6, 3M solution) with equal volume of Isopropanol was combined in the tubes. They were then inverted many times to precipitate the DNA and centrifuged at 13,000rpm for another 10 minutes.

iv. The acquired supernatant after centrifugation was then cast away and the remaining pellet was rinsed with 200µl of 100% Ethanol. After the ethanol wash

the tubes were centrifuged at 13,000rpm for 7 minutes and ethanol was thrown away afterwards.

v. The pellet was washed again with 200µl of 70% Ethanol and centrifuged at 13,000rpm for 7 minutes.

vi. The Ethanol was then discarded, and the sample was dried for 30-40 minutes at room temperature to evaporate Ethanol completely.

vii. The samples were then added with 200µl of TE buffer to dissolve and preserve the DNA pellet. The pellet containing tubes were stored at -20ºC.

Table 2: Solutions for DNA Extraction

<table>
<tr><th>Solution A (Blood cell lysis)</th><th>Solution B (DNA & Protein Precipitation)</th><th>Solution C (DNA Isolation)</th><th>Solution D (DNA Purification)</th></tr>
<tr><td>0.32M Sucrose</td><td>10mM Tris (pH7.5)</td><td rowspan="4">Phenol</td><td rowspan="2">Chloroform (24 Volume)</td></tr>
<tr><td>10mM Tris (pH 7.5)</td><td>400mM NaCl</td></tr>
<tr><td>5mM $MgCl_2$</td><td rowspan="2">2mM EDTA (pH 8)</td><td rowspan="2">Isoamyl alcohol (1 volume)</td></tr>
<tr><td>Triton X-100 1% (v/v)</td></tr>
</table>

3.5. Gel Electrophoresis of DNA (1%)

To analyse the quality of genomic DNA 1% (w/v) gel agarose is used. The following procedure was employed:

i. 1g of agarose powder was weighed on an electronic weighing balance and was solvated in 100ml of 1X of TBE buffer in a microwave for approx. 2 minutes.
ii. After the solution steamed off for a while, ethidium bromide (8µg/ml) was mixed into the gel mixture for the purpose of staining.
iii. The mixture of gel was then poured into a gel casting tray and was put to rest at room temperature to solidify.
iv. The gel loading was done by placing the gel in a gel tank which contained 1X TBE buffer. A mixture of loading dye and genomic DNA was deposited into the gel wells.
v. The gel was electrophoresed at 90V for 45 minutes and it was then analyzed in Omnidoc and an electrogram of the gel were shot.

3.6. Quantification of DNA

The extracted genomic DNA's quantification was acquired through ThermoScientific Nanodrop 2000 UV-Vis Spectrophotometer and NanoDrop 2000™ software at the ASAB laboratory. The TE buffer was used as a blank and later the sample was loaded to observe the absorbance ratio. A standard wavelength of 260nm was taken as optimum absorption of nucleic acid and was recorded at it. A wavelength ratio for DNA purity was taken at 260/280nm.

3.7. Primer Designing

Gene sequences of *VEGF-A rs699947* with CA polymorphism is situated at chromosome 6:43768652 (GRCh38.p12) and *VEGF-A rs2146323* is located at chromosome 6:43777358 (GRCh38.p12) with CA polymorphism. Their sequence of

genes was obtained from National Centre for Biotechnology Information (NCBI) Gene Bank for the tetra primer designing by the aid of Primer3 online software in combination with Primer Blast software. Online software OligoCalc was employed to check the complementarity of the primer with the required sequence and formation of any probable hairpin structures. Online in-silico PCR under the UCSC browser was used to gauge the probable specificity and efficacy of the Primers.

Table 3: Tetra Primer Sequences for Allele Specific PCR

S. No.	Gene	Sequence (5'-3')	Base pairs	Amplicon size
1.	VEGF-A 699947 Forward C	CAGCTGTAGGCCAGACCCTGGCAC	24	377bp
2.	VEGF-A 699947 Reverse C	TCTATCAGCCCAAGCCCAGACT	22	377bp
3.	VEGF-A 699947 Forward A	CAGCTGTAGGCCAGACCCTGGCAA	24	210bp
4.	VEGF 699947 Reverse A	AACTCTCCACATCTTCCCTAAGTGCT	25	210bp
5.	VEGF-A 2146323 Forward C	AGGGACTTACGTTAGATTTTGGAAGGAC	28	187bp
6.	VEGF-A 2146323 Reverse C	TCGATTGGATGGCAGTAGCTG	21	187bp
7.	VEGF-A 2146323 Forward A	AGGGACTTACGTTAGATTTTGGAAGGAA	28	332bp
8.	VEGF-A 2146323 Reverse A	CATGGTGATGTTGGACTCCTCAG	23	332bp

3.8. Polymerase Chain Reaction (PCR)

The samples of the subjects under the study were put through allele specific polymerase chain reaction to amplify the target SNPs and to analyze their polymorphism in a certain set of population. Tetra primers were designed for the polymorphism analysis, so a set of reverse and forward primers were prepared and incorporated into a single tube. Two sets of tubes for each set of tetra primers set were utilized. Amplification of the designated primer set indicated the presence of the required polymorphism. The following table shows the reaction mixture profile optimized for 20µl for the *VEGF-A rs699947* and *VEGF-A rs2146323* PCR. The reaction mixture's preparation was always done in a Biosafety Cabinet available at Immunogenetics Lab, ASAB, and the reaction mix was spun for 30seconds for homogenization of contents and removal of air bubbles. The tubes with the reaction mix in them were loaded in a Thermocycler 2720 (Applied Biosystems, Foster City, USA). The optimized conditions for the Thermocycler are given in the following figures.

Table 4: Reaction mixture profile of VEGF-A rs699947 and VEGF-A rs2146323

REAGENTS	VEGF-A rs699947	VEGF-A rs2146323
Genomic DNA (50ng/µl)	1µl	1µl
PCR Buffer	2µl	2µl
$MgCl_2$	2µl	2µl
10mM DNTPs	1µl	1µl
Forward Primer	1µl	1µl
Reverse Primer	1µl	1µl
Taq DNA polymerase	0.25µl	0.25µl
Nuclease free water	11.75µl	11.75µl

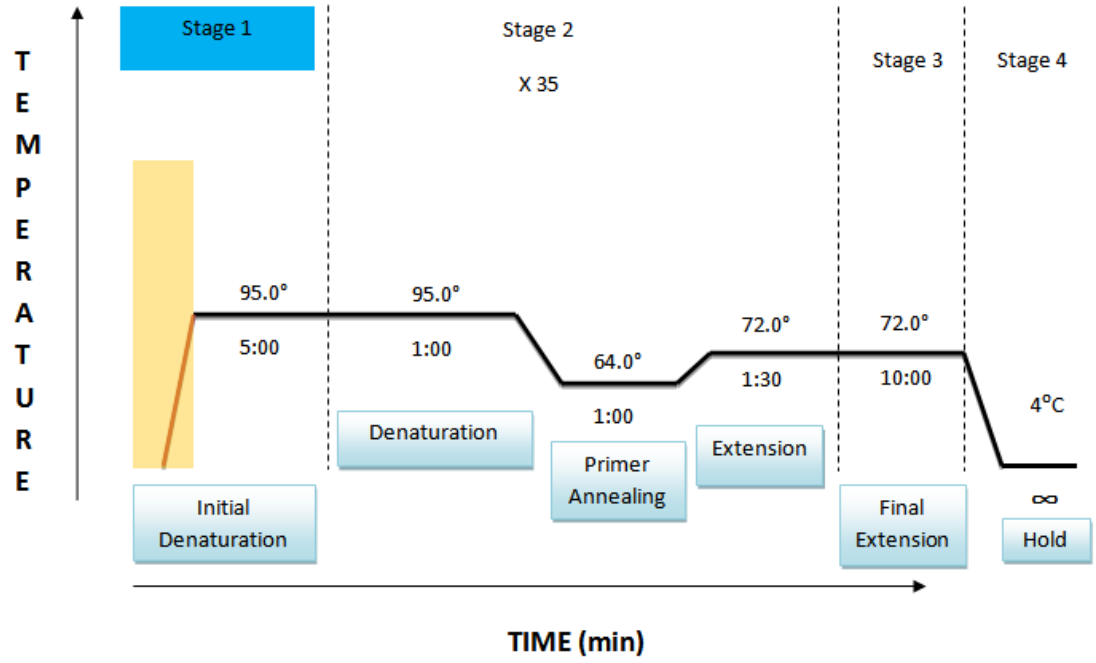


Figure 6: PCR Profile for VEGF-A rs699947

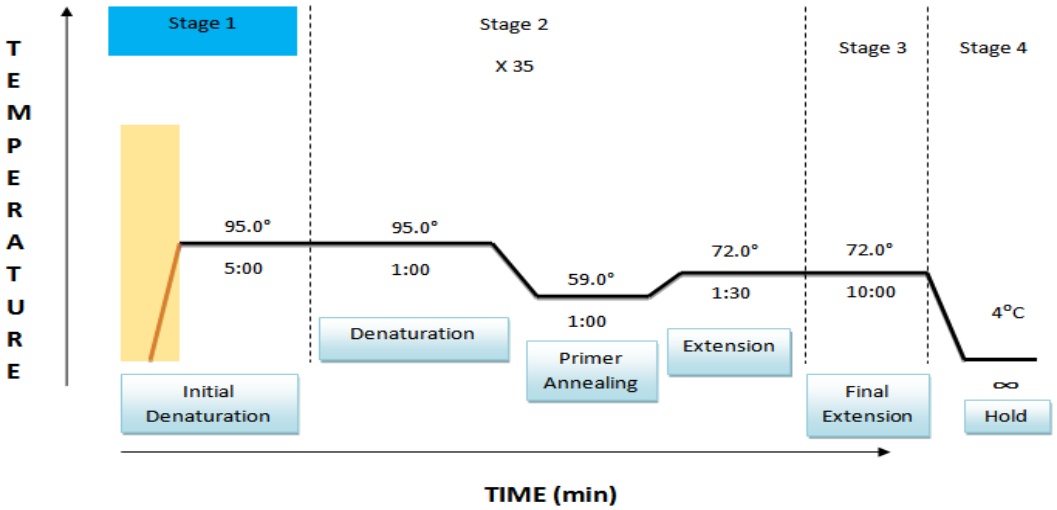


Figure 7: PCR Profile for *VEGF-A rs2146323*

3.8.1. Gel Electrophoresis for PCR Products 2% (w/v)

For the analysis of PCR products, 2% w/v gel was primed by dissolving 2g of agarose powder in 100ml of 1X of TBE buffer and was heated in the microwave. 5µ/ml of ethidium bromide was poured to agarose solution, after its steam-letting. The mixture was then poured into a casting tray for solidification of the gel at room temperature. The solidified gel was positioned in the electrophoresis container and the loading dye (bromophenol blue) and the PCR products in a ratio of 2:5 were loaded in the gel wells through the micropipette along with the 100bp ladder to classify size of the bands. The gel electrophoresis was optimized at 85V for 45 minutes. The electrogram was shot under the Omnidoc to analyze the bands.

3.9. Statistical Analysis of the Samples

GraphPad Prism and online 2x2 contingency table was employed to calculate statistical functions as Chi-Square, Odds Ratio, Genotypic Frequency, Relative Risk and Allelic Frequency. P-values of *P<0.05, **P<0.005, ***P<0.001 were mediated as significant.

Chapter 4

Results

4.1. Analysis of Collected Samples

The samples of blood from T2DM subjects and from unimpaired controls were collected for the genomic DNA extraction. The sample collection was done from Military Hospital, Rawalpindi; PIMS, Islamabad; Amna Hospital, Sialkot and Sialkot Medical Complex, Sialkot. A total of 186 samples were collected (T2DM: 106 samples, Controls: 80).

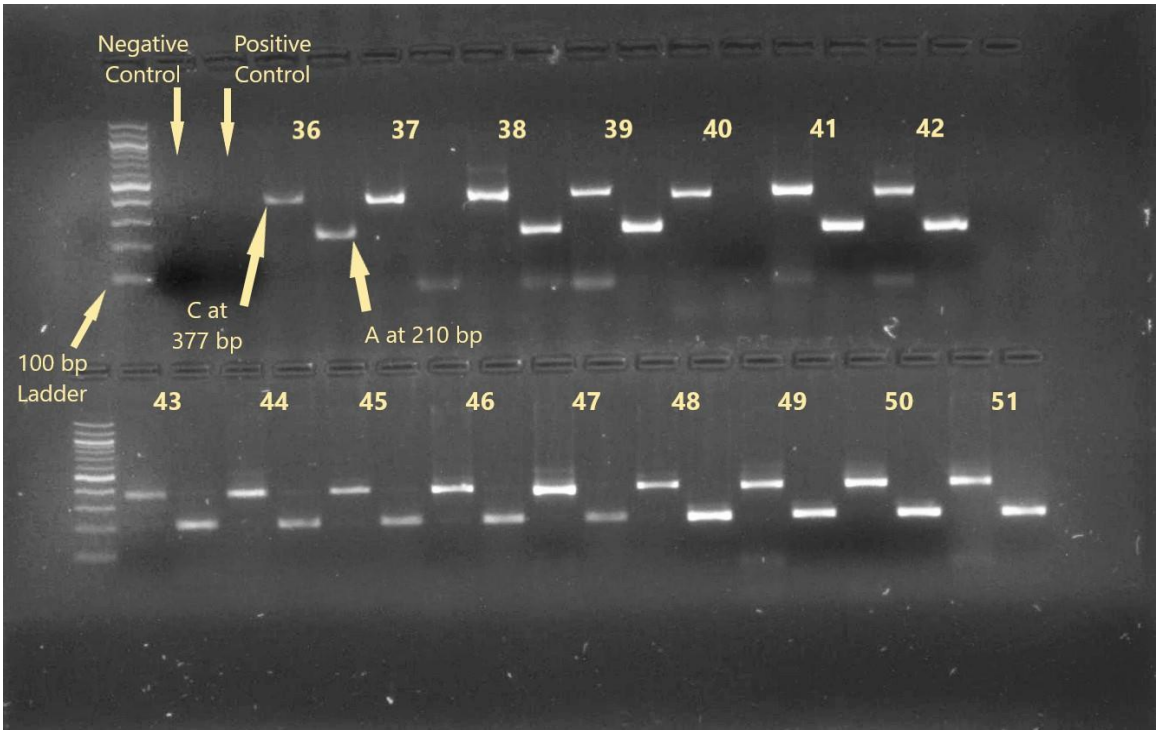
4.2. Genomic DNA Extraction

Phenol-Chloroform® method was employed to take out DNA from the blood samples. The protocol was two days long. To determine the quantity and quality of the DNA, ThermoScientific NanoDrop 2000 Spectrophotometer and NanoDrop™ 2000 software was used. The optimum wavelength of 260/280nm was recorded to determine the purity of the extracted DNA in the samples.

4.3. Association Analysis of VEGF-A rs699947 and VEGF-A rs2146323

Association analysis of *VEGF-A rs699947* and *VEGF-A rs2146323* was performed using polymerase chain reaction. All the enrolled subjects in the study were screened for both the polymorphisms. The ethidium bromide stained

electropherogram of 2% agarose gel with the PCR products of SNPs i.e. *VEGF-A rs699947* and *VEGF-A rs2146323* are given in the (Figure 8) and (Figure 9) respectively. The *VEGF-A rs699947* SNP is located in the promoter region with an SNV (Single nucleotide variation) of C>A. The genotypic frequency is illustrated in Figure 10 and 11 and allelic frequency is given in the graph in Figure 12 and 13 for the experimental vs. control groups. *VEGFA rs2146323* is an intron variant located at chromosome 6 at position chr6:43777358 It possesses the C>A as SNV.



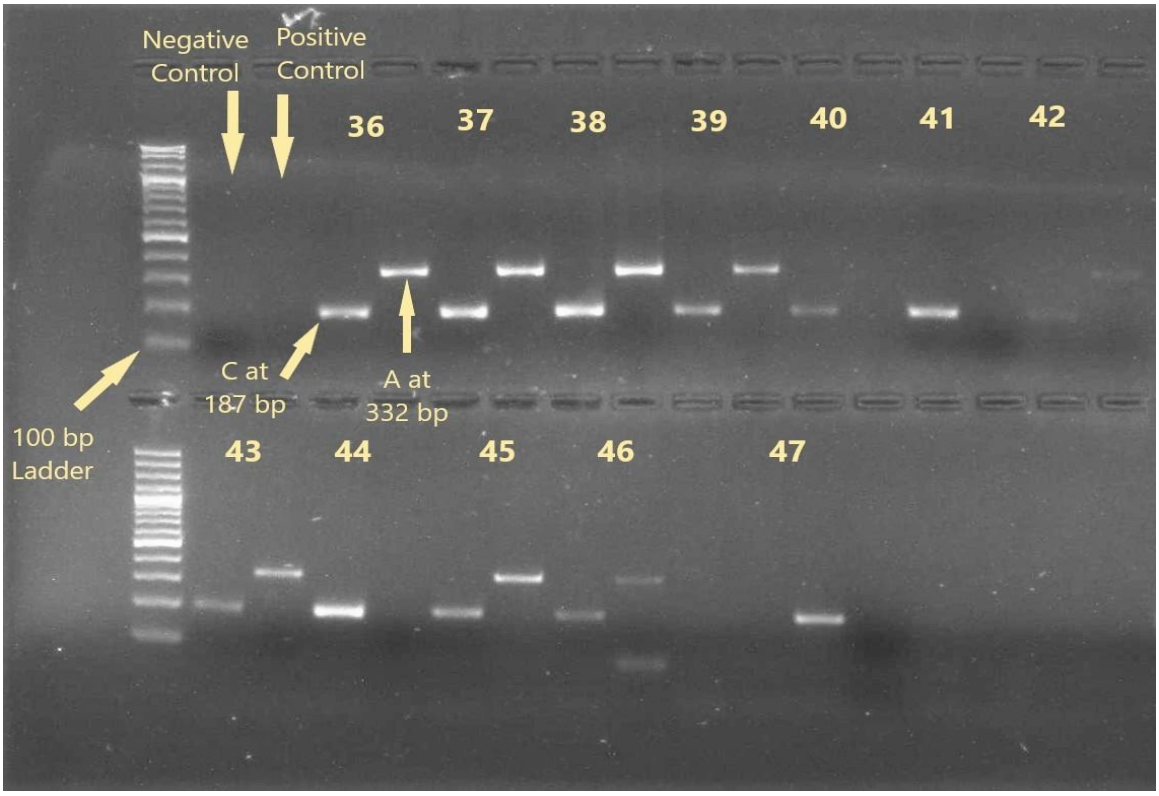
L: Ladder (100bp)

Diabetes Samples: 36 - 51

C Amplicon Size: 377bp

A Amplicon Size: 210bp

Figure 8: VEGF-A rs699947 electropherogram of amplified PCR products on 2% agarose gel stained with ethidium bromide



L: Ladder (100bp)

Diabetes Samples: 36 - 47

C Amplicon Size: 187bp

A Amplicon Size: 332bp

Figure 9: VEGF-A rs2146323 electropherogram of amplified PCR products on 2% agarose gel stained with ethidium bromide

The products of PCR for VEGF-A rs699947 and rs2146323 SNP was loaded on 2% agarose gel stained with ethidium bromide for the purpose of analysis along with 100bp ladder to tally the band size and positive and negative controls. The first band indicates the heavier C allele at 337bp while the second band symbolizes A allele at an Amplicon size of 210bp for *rs699947* in figure 8. For *rs2146323* in figure 9, the first lighter band at 187bp indicates C allele and the heavier band at 332bp indicates the A allele.

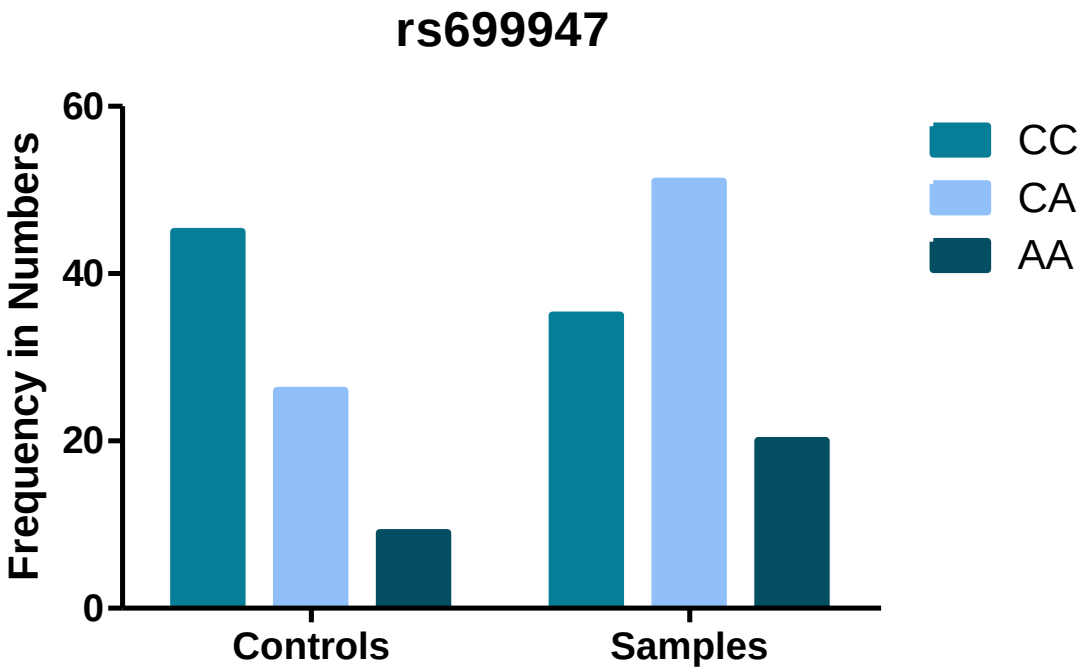


Figure 10: Genotypic distribution of VEGF-A rs699947 polymorphism in experimental vs. control groups

The genotypic frequency graph of VEGF-A rs699947 depicts that CA the heterozygous genotype is found prevalently in samples of T2DM as compared to homozygous genotype of CC in Controls. The highest ratio of CA was found in T2DM patient samples.

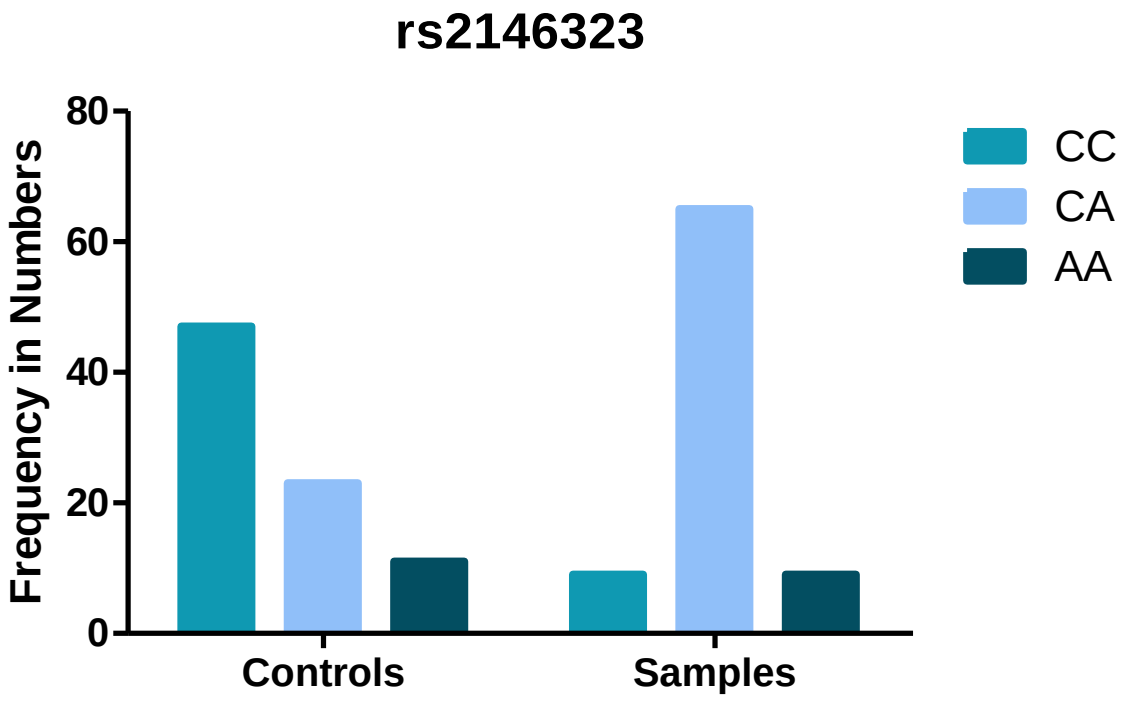


Figure 11: Genotypic distribution of VEGF-A rs2146323 polymorphism in experimental vs. control groups

The genotypic graph of rs2146323 shows that the heterozygous genotype CA stands out with the highest ratio in T2DM samples as compared to control samples. The frequency of AA and CC genotype is marginally equal in T2DM samples yet in controls the normal genotype of CC is more prevalent.

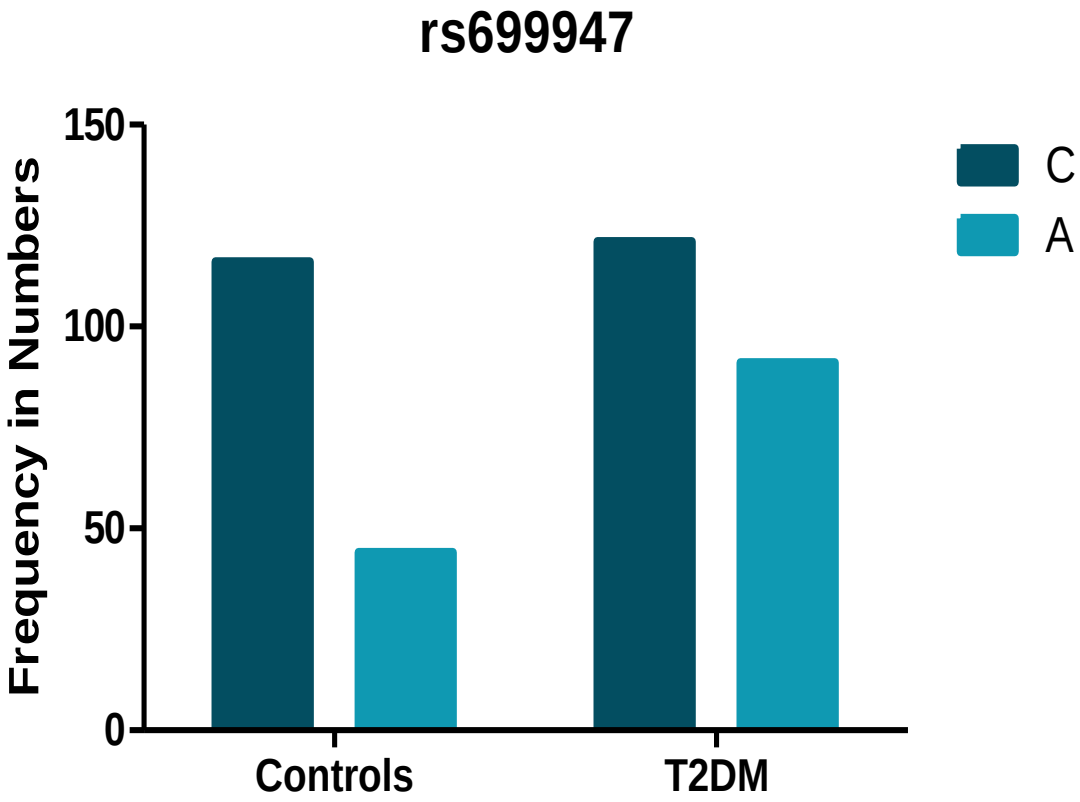


Figure 12: Allelic distribution of VEGF-A rs699947 polymorphism in experimental vs. control groups

The allelic frequency graph shows that the C allele and A allele have a less distributive difference in T2DM samples since they both exist as heterozygous genotype in abundance in T2DM samples. In controls the C wild-type allele is more abundant.

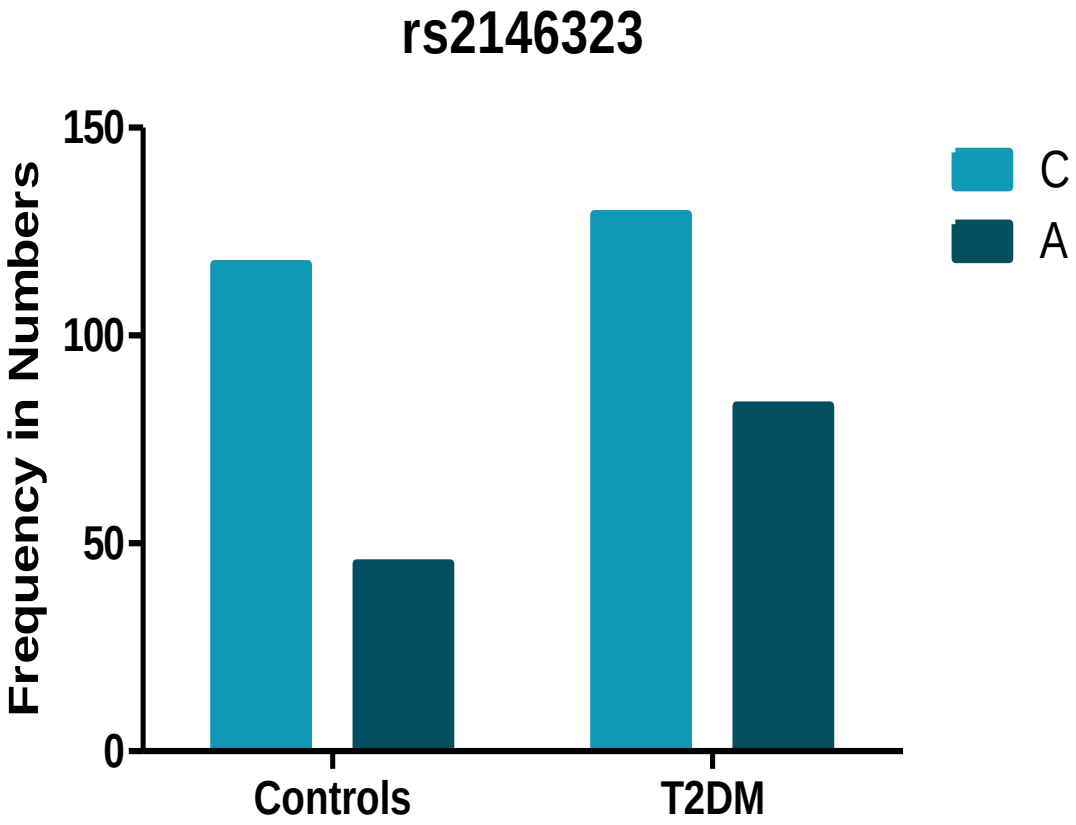


Figure 13: Allelic distribution of VEGF-A rs2146323 polymorphism in experimental vs. control groups

The allelic frequency graph of rs2146323 shows that the C allele is more prevalent in T2DM and Control samples. The A allele is less prevalent.

Table 5: Genotype and allele frequencies of VEGF-A rs2146323 and VEGF-A rs699947 polymorphisms

SNP	Control	T2DM		Control vs. T2DM				
	Number (%)	Number (%)	OR (95%CI)	RR (95%CI)	P Value	Chi Square	Chi Square	P Value
rs2146323								
Genotype								
CC	47 (58%)	32 (30%)	0.3 0.17-0.6	0.5 0.3-0.7	0.000134	14.58	46.1	<0.0001***
CA	23 (28%)	65 (61%)	3.9979 2.1-7.4	2.15 1.48-3.1	<0.0001	19.98		
AA	11 (14%)	9 (8%)	0.59 0.23-1.5	0.6 0.2-1.5	0.26	1.25		
Allele								
C	117 (72.2%)	129 (61%)	0.59 0.38-0.92	0.8 0.72-0.97	0.02	5.28	0.0276	0.0277
A	45 (27.8%)	83 (39%)	1.67 1.07-2.59	1.4 1.07-2.59	0.02	5.28		
rs699947								
Genotype								
CC	45 (56%)	35 (44%)	0.38 0.21-0.69	0.58 0.42-0.81	0.021	5.28	10.10	0.0064**
CA	26 (33%)	51 (64%)	1.9 1.05-3.52	1.4 1.01-2.14	0.03	4.58		
AA	9 (11%)	20 (25%)	1.8 0.78-4.28	1.67 0.80-3.48	0.15	2.01		
Allele								
C	116 (72.5%)	121 (57%)	0.5 0.32-0.78	0.78 0.67-0.91	0.0021	9.38	8.73	0.0023
A	44 (27.5%)	91 (43%)	1.98 1.27-3.08	1.56 1.16-2.09	0.0021	9.38		

OR = Odds Ratio
RR = Relative Risk
95% CI, Confidence Interval
P= Probability Value
chi square x^2-test and Fisher-exact test for genotype and for allele frequencies
Controls: 81
T2DM: 106

4.4. Association of VEGF-A rs2146323

A strong association was found between VEGF-A SNP rs2146323 and T2DM. The heterozygous genotype CA with an OR=3.99, 95% CI= (2.1-7.4), P=0.0001*** indicates a susceptibility of developing DR in T2DM patients. The genotype CC hinted at a protective effect against DR in T2DM suffering patients with an OR=0.3, 95% CI= (0.17-0.6), P= 0.000134. The genotype AA may put the patient at slight possibility of developing DR with uncontrolled T2DM, an OR=0.59, 95% CI= (0.23-1.5), P= 0.26 was calculated. The chi (x^2) value (46.1) with P= (<0.0001***) suggests a strong affiliation between the VEGF-A SNP rs2146323 and the T2DM patients' risk of developing DR.

The allelic frequency graph indicates that the A allele may pose as a risk allele which may have a more potent effect heterozygous CA and increased risk in homozygous AA genotype.

4.5. Association of VEGF-A rs699947

A significant association was observed in the limited sample pool between VEGF-A rs699947 and the development of DR as a co-morbidity of T2DM. The heterozygous genotype CA with OR=1.9, 95% CI= (1.05-3.52), P= 0.03 increases the possibility of DR in diabetic patients. The homozygous genotype AA with OR=1.8, 95% CI= 0.78-4.28), P= 0.15 also slightly increases the possibility of developing Diabetic Retinopathy for diabetic patients. Whereas, the homozygous genotype CC with the OR= 0.38, 95% CI= (0.21-0.69), P= 0.02, has a protective effect against DR

in T2DM patients. The chi (x^2) value 10.10 with P= 0.0064** suggests a substantial correlation of developing DR in diabetic patients.

The allelic graph shows that the allele A poses a raised risk of DR development along with T2DM. A is the ancestral allele for this variant. The allele C has a shielding effect. Any discrepancies may be due to the limited number of the samples in the pool.

Chapter 5

Discussion

Type 2 Diabetes mellitus (T2DM) or Non-insulin Dependent Diabetes Mellitus (NIDDM) is a disease outlined by the gradual loss of activity of β-cells in the pancreas and hence the development of chronic resistance to insulin which eventually causes the buildup of sugar circulating in the bloodstream. T2DM is a disease that is strongly related with and is a root cause of various co-morbidities. One of them is Diabetic Retinopathy (DR). DR stands out as the chief cause of blindness worldwide. The diabetic population suffers greatly through Diabetic Retinopathy. It is a case of concern for ophthalmologist world over to control and possibly stop the progression of DR before it takes away the sight of the patient. Vascular Endothelial Growth Factor with isoform A, erstwhile known as VEGF-A has been closely linked with triggering DR in T2DM patients in cohort studies conducted over different ethnic populations. It is a drastic effect of long-standing, uncontrolled Diabetes. Close to 150 million people world over are affected by DR. And the number may possibly double by the year 2025. 24% of patients can possibly acquire any form DR among the diabetic population. The VEGF-A molecule is popularly ingrained agent in the development of DR. It alters the permeability of retinal capillaries by upping the rate of phosphorylation of proteins in the tight-junction spots such as zonula occludens. Mitogen activated proteins (MAP) are resultantly triggered and cause endothelial proliferation of cells. VEGF-A is also responsible for stirring endothelial cells to release MMPs which ultimately results in the basement membrane degradation and making migration of cell possible. Endothelial cell migration and proliferation ends

up in formation of basic membranes for the recently integrated capillaries causing the thickening of the retina or DR.

In this study, two of the three most closely associated variants of VEGF-A to DR in T2DM patients; *rs2146323* and *rs699947* were selected to study among a limited sample pool of Pakistani T2DM patients. Both the SNPs reside on chromosome 6. An allele specific PCR study was conducted to register the allelic and genotypic frequencies of the above-mentioned SNPs of VEGF-A in the blood samples of T2DM patients in respect to DR.

VEGF-A rs699947 genotype (CA) was observed in relation with T2DM signaling its involvement in the proliferation of DR. VEGF-A rs2146323 genotype (CA) was gravely associated with T2DM patients increasing the odds of DR among the limited sample pool of patients employed in the study. The polymorphisms rs699947 and rs2146323 might not entirely be responsible for stimulating the pathogenesis of DR in T2DM patients but could be indicative of certain phenotypes and their progression. To fully understand the import of involvement of VEGF-A polymorphisms in DR a thorough investigative research is required to highlight its role in disease pathogenesis and its complications. A study composed to research over a much larger sample pool along with in-silico analysis indicating phenotypic features and possible drug targets is required.

Conclusion

VEGF-A *rs2146323* CA genotype was found to be closely connected with T2DM and a plausible incidence of DR in the limited sample study. VEGF-A *rs699947* CA genotype significantly increases the risk ratio of DR in T2DM patients. The relationship between these variants suggests that they may portray a role in etiology and disease progression of DR in subjects. However, the findings of this study needs to be replicated on a large scale of patients' sample and in other populations to be well endorsed. The allelic distribution of both SNPs is indicative of no significant difference of alleles in T2DM samples and Controls.

Future Prospects

To enhance the understanding of the VEGF-A variants' model of DR in T2DM patients, expressional studies could be conducted to estimate the conformation of expression in T2DM and DR as compared to control samples. VEGF-A rs2146323 and rs699947 might have substantial role in disease pathogenesis but a study conducted on larger sample size to obtain large-scale data would be required, only then its role in disease etiology would be verified. Large sample size and data study will be useful in designing apt *in-silico* drug targets for DR. Both these SNPs of VEGF-A could be identified as prognostic markers for the progression of the disease. Discerning the pathways and functions of VEGF-A in various healthy and diseased states may aid in the identification of therapeutic approaches that may hinder the detrimental effects of VEGF-A variants while preserving their favorable functions.

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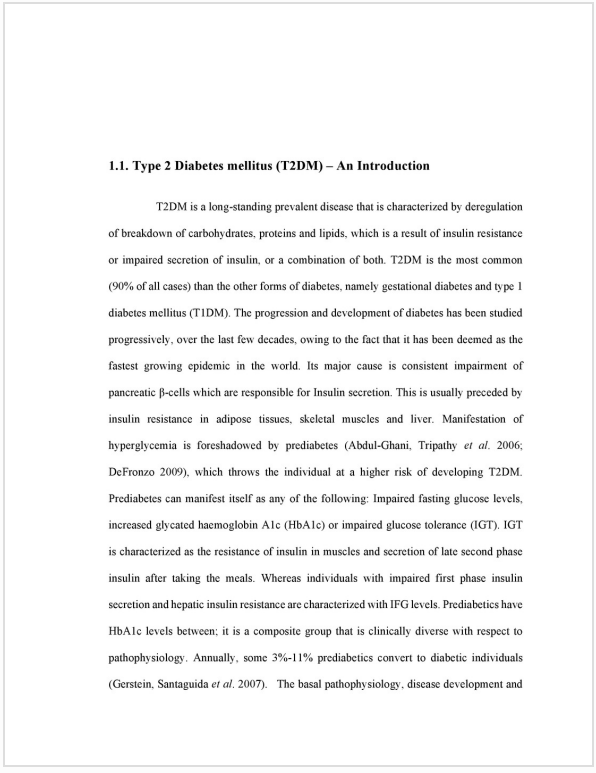

1.1. Type 2 Diabetes mellitus (T2DM) – An Introduction

T2DM is a long-standing prevalent disease that is characterized by deregulation of breakdown of carbohydrates, proteins and lipids, which is a result of insulin resistance or impaired secretion of insulin, or a combination of both. T2DM is the most common (90% of all cases) than the other forms of diabetes, namely gestational diabetes and type 1 diabetes mellitus (T1DM). The progression and development of diabetes has been studied progressively, over the last few decades, owing to the fact that it has been deemed as the fastest growing epidemic in the world. Its major cause is consistent impairment of pancreatic β-cells which are responsible for Insulin secretion. This is usually preceded by insulin resistance in adipose tissues, skeletal muscles and liver. Manifestation of hyperglycemia is foreshadowed by prediabetes (Abdul-Ghani, Tripathy *et al*. 2006; DeFronzo 2009), which throws the individual at a higher risk of developing T2DM. Prediabetes can manifest itself as any of the following: Impaired fasting glucose levels, increased glycated haemoglobin A1c (HbA1c) or impaired glucose tolerance (IGT). IGT is characterized as the resistance of insulin in muscles and secretion of late second phase insulin after taking the meals. Whereas individuals with impaired first phase insulin secretion and hepatic insulin resistance are characterized with IFG levels. Prediabetics have HbA1c levels between; it is a composite group that is clinically diverse with respect to pathophysiology. Annually, some 3%-11% prediabetics convert to diabetic individuals (Gerstein, Santaguida *et al*. 2007). The basal pathophysiology, disease development and

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