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Identification of TCF7L2 Genetic Variants Associated with Type 2 Diabetes in Northern Region of Bangladesh

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University of Rajshahi

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IDENTIFICATION OF *TCF7L2* GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES IN NORTHERN REGION OF BANGLADESH

PhD Thesis

A Dissertation

Submitted to the Institute of Biological Sciences, University of Rajshahi for Partial Fulfillment of the Requirement towards the Doctor of Philosophy (PhD) Degree on Biochemistry

Submitted by

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August, 2018

Dedicated

to

My Family

Declaration

I hereby declare that the entire research work entitled "IDENTIFICATION OF *TCF7L2* GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES IN NORTHERN REGION OF BANGLADESH" is now submitted as a thesis to the Institute of Biological Sciences (IBSc), University of Rajshahi towards the partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) on Biochemistry has been given.

…………………………….....

DIPA ROY

Certificate

We hereby certify that the thesis work entitled **"IDENTIFICATION OF *TCF7L2* GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES IN NORTHERN REGION OF BANGLADESH"** has been prepared under our direct supervision and guidance by the candidate bearing Roll No. P-504, Reg. No. 0163, session 2014-2015 and examination-2018 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) from the Institute of Biological Sciences, University of Rajshahi, Bangladesh.

It is also certified that the research work embodied in this dissertation is original and carried out by **DIPA ROY** and the work or part of it has not been submitted before as candidature for any other degree.

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August, 2018 *Dipa Roy*

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Abbreviations

ABCC8: ATP-binding cassette transporter subfamily C member 8

ADA: American Diabetes Association

ARMS: Amplification-refractory mutation system

ATP: Adenosine triphosphate

BIRDEM: Bangladesh Institute of Research and Rehabilitation for Diabetes,

Endocrine and Metabolic Disoreders

BMI: Body mass index

CAPN10: Calpain10

CDKAL1: CDK5 regulatory subunit associated protein 1-like 1

CVD: Cardiovascular disease

DKA: Diabetic ketoacidosis

DHPLC: Denaturating high performance liquid chromatography

DM: Diabetes mellitus

DPP: Diabetes Prevention Program

DKA: Diabetic ketoacidosis

DMSO: Dimethyl sulfoxide

DNp: Diabetic nephropathy

DNu: Diabetic neuropathy

DR: Diabetic retinopathy

DPP-IV: Dipeptidyl peptidase IV enzyme

dsDNA: Double stranded DNA

E23K: Association of KCNJ11

ENPP1 **:** Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1

FPG: Fasting plasma glucose

FTO: Fat mass and obesity-associated protein

GDM: Gestational diabetes-mellitus

GLP-1: Glucagon-like peptide-1

GAD: Glutamic acid decarboxylase

GWA: Genome-Wide Association

HbA1c: Glycated haemoglobin

HRM: High resolutions melt

HGP: Human Genome Project

HMG: High-Mobility Group

HHEX: Hematopoietically-expressed homeobox protein

HHNS: Hyperosmolar hyperglycemic nonketotic syndrome

ICAs: islet cell cytoplasmic antibodies

IGF2BP2: Insulin Like Growth Factor 2 MRNA Binding Protein 2

JAZF1: Juxtaposed with another zinc finger protein 1

KCNJ11: ATP-sensitive inward rectifier potassium channel 11

LADA: Latent autoimmune diabetes in adults

LD: Linkage Disequilibrium

mABs : Monoclonal antibodies

MODY : Maturity-Onset Diabetes of the Young

NIDDM: Non-insulin-dependent diabetes mellitus

NOTCH2: Neurogenic Locus Notch Homolog Protein 2

OD: Optical density

OGTT: Oral glucose tolerance test

OHA: Oral hypoglycemic agents

PPARG: Peroxisome proliferator-activated receptor-gamma

POS: Polycystic ovary syndrome

qPCR : Quantitative polymerase chain reaction

RPGT: Random plasma glucose test

Real-time PCR: Real-time polymerase chain reaction

RBC: Red blood cell

RFLP: Restriction fragment length polymorphism

SNPs: Single nucleotide polymorphisms

SSCP: Single Strand Conformational Polymorphism Analysis

ssDNA: Single stranded DNA

SLC30A8: Solute carrier family 30 (zinc transporter), member 8

Tg : Triglycerides

TCF7L2: Transcription Factors 7-Like 2

TCF/LEF: T-cell factor/lymphoid enhancer factor

T1D: Type 1 diabetes

T1DM: Type 1 diabetes mellitus

T2D: Type 2 diabetes

T2DM: Type 2 diabetes mellitus

Tm: Melting Temperature

WBC: White blood cells

WFS1: Wolfram Syndrome 1

WHO: World Health Organization

Abstract

Background: Seemingly harmless nucleotide changes are found in sequence scanning but their possible association with diseases often remains unclear. However, the transcription factor-7-like 2 (*TCF7L2*) gene has been identified as a major risk associated gene for type 2 diabetes mellitus (T2DM) susceptibility. Identified intronic locus of the gene harbor five diabetes associated polymorphisms including rs7901695. rs7901695 polymorphism has been previously reported to be associated with diabetes type 2 in several ethnic groups but, no genetic analysis has been carried out to show the relationship of rs7901695 with type 2 diabetes in Bangladeshi population.

Objective: This study was aimed to investigate the association between T2DM and *TCF7L2* variants in Bangladeshi population.

Materials and methods: In this research work, 657 individuals from northern region of Bangladesh were analyzed. Out of those, 330 were non-diabetic served as control and 327 were T2DM individuals. For genotyping of *TCF7L2* variants, we have used High Resolution Melting (HRM) analysis. Demographic risk factors like body mass index (BMI), triglyceride (Tg), glycalated hemoglobin (HbA1c) and total cholesterol were also investigated routinely. Significance of these risk factors was further analyzed statistically.

Results: Allelic frequency of the participants differed significantly ($P < 0.05$) between T2DM and non-diabetic control. Patients with T2DM had significantly higher values for BMI, Tg, HbA1c and total cholesterol. This study revealed 87 mutations out of 327 T2DM patients while no mutation was detected in healthy person. However, in the allelic distribution of rs7901695, we have found allele type AA in 139 patients and GG in 101. Whereas, polymorphic heterozygous AC in 11 and GC in 23 and homozygous CC in 41 and TT in 12 patients respectively.

Conclusion: This study suggests that *TCF7L2* genetic variants are closely correlated with T2DM in population of the Northern region of Bangladesh.

Keywords: *TCF7L2*; polymorphism; type 2 diabetes; Bangladeshi population.

Chapter 1

Introduction

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas. In other words, it could be defined as the ineffectiveness of the insulin produced. This type of deficiency results in increased concentrations of glucose in the blood that in turn damage many of the body's systems, particularly the blood vessels and nerves. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels (American Diabetes Association, 2014). DM is the single most important metabolic disease, widely recognized as one of the leading causes of death and disability worldwide (Zimmet, 1982). The World Health Organization (WHO) estimated that the total number of people with DM is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004).

DM is now one of the most common non-communicable diseases globally. It is epidemic in many developing and industrializing countries. China and India hold the first and second position respectively having 98.4 and 65.1 million of total cases of DM in adult population (20 to 79 years) in 2013. According to prevalence rate Tokelau and Micronesia hold the first and second position respectively having 37.5% and 35.0% diabetics in adult population in 2013 (L'Heveder & Nolan, 2013).

These days, one-fifth of all DM people of the world reside in the South-East Asia region. (International Diabetes Federation, 2011) As a part of South-East Asia, in Bangladesh the magnitude of DM is also rising. Here, in the mid 70s the rate of DM was around 1.0 to 1.5%, which has increased to 15% recently (L'Heveder & Nolan, 2013).

Currently, 366 million people have diabetes worldwide, and the number is predicted to reach 552 million by 2030 (Shikata et al., 2013). Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder in which prevalence has been increasing steadily all over the world (Olokoba et al., 2012). T2DM is characterized by peripheral insulin resistance in the liver, skeletal muscle, and adipose tissues, as well as impaired insulin secretion from the pancreatic β-cells (Meshkani et al., 2012). Evidence has been presented that T2DM drives from the coexistence of genetic and environmental factors (Golshani et al., 2015). Many genes are believed to contribute to the pathophysiology of T2DM through multiple

biological pathways. Linkage studies have provided little success in identifying genes for T2DM. So far more than 50 genome-wide linkage scans have been performed to map the genes for T2DM susceptibility in a variety of populations (Barroso, 2005).

The transcription factor 7-like 2 gene (*TCF7L2*) is one of the most convincing susceptibility genes for type 2 diabetes. The role of *TCF7L2* in the pathogenesis of T2DM has been investigated in several studies. Following the initial report (Grant et al., 2006), five single nucleotide polymorphisms (SNPs) within introns 3 and 4 of the *TCF7L2* gene (rs12255372, rs7903146, rs7901695, rs11196205, rs7895340) were identified to associate with an increased risk of type 2 diabetes. Recently, variants in the *TCF7L2* gene also were reported to be associated with β-cell function (Schäfer et al., 2007; Lyssenko et al., 2007) and response to sulfonylureas in Caucasians (Pearson et al., 2007).

The relationship between *TCF7L2* and T2DM in Bangladesh has not yet been addressed. Results from other populations have been adopted to explore the possible association between this polymorphism and T2DM and its metabolic quantitative traits in a sample of the Bangladesh. The allele and genotype frequencies of above polymorphism in people with and without T2DM, and detected their effects on anthropometric, diabetes and obesity-related parameters.

1.1 Overview of Diabetes Mellitus (DM)

Diabetes mellitus (DM) is a complex heterogeneous group of disorders characterized by persistent hyperglycemia and caused by an absolute or relative deficiency of insulin, which is an anabolic hormone, produced by the beta cells of the islets of Langerhans located in the pancreas. The World Health Organization (W.H.O) (WHO/IDF, 1999 and 2006) describes DM as a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, action or both, the American Diabetic Association (ADA) (ADA, 2004, 2008 and 2009). Defines DM as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both.

Insulin plays a critical role in balancing glucose levels in the body. Insulin can inhibit the breakdown of glycogen or the process of gluconeogenesis. Insulin is released into the blood by beta cells (β-cells), found in the islets of Langerhans in the pancreas, in response to rising levels of blood glucose, typically after eating. Insulin is used by about two-thirds of the body's cells to absorb glucose from the blood for use as fuel, for conversion to other needed molecules, or for storage. Lower glucose levels result in decreased insulin release from the β-cells and in the breakdown of glycogen to glucose. This process is mainly controlled by the hormone glucagon, which acts in the opposite manner to insulin.

If the amount of insulin available is insufficient, if cells respond poorly to the effects of insulin (insulin insensitivity or insulin resistance), or if the insulin itself is defective, then glucose will not be absorbed properly by the body cells that require it, and it will not be stored appropriately in the liver and muscles. The net effect is persistently high levels of blood glucose, poor protein synthesis, and other metabolic derangements, such as acidosis.

1.2 History of Diabetes mellitus

Diabetes mellitus as a disease, for example a constellation of symptoms, but not its pathogenesis, has been known by physicians for nearly 3,500 years in ancient Egypt (Ebbell et al., 1937). The Ebers papyrus dating from 1550 BC was found in a grave in Thebes region south of Egypt in 1862, and named after the Egyptologist Geary Ebers (Ebbell et al., 1937). The papyrus contains descriptions of various diseases, among them is a polyuric syndrome, presumably diabetes. The Egyptians suggested various remedies to this syndrome including a decoction of bowes, wheat and earth (Ebbell et al., 1937). The Verdic medical treatises from ancient India described, in detail, diabetes like conditions of 2 types: Congenital and late onset (Algaonker, 1972). Also, the Indians noticed the relation of diabetes to heredity, obesity, sedentary life and diet. They suggested the freshly harvested cereals and bituminous preparations containing benzoates and silica as a remedy for diabetes. The first time association of polyuria with a sweet -tasting substance was reported in the Indian literature from the 5th-6th century AD by Sushrant (a notable Indian physician) (Algaonker, 1972).

For 2,000 years diabetes has been recognized as a devastating and deadly disease. In the first century A.D. a Greek physician, Aretaeus, described the destructive nature of the affliction, which he named "diabetes" from the Greek word for "Siphon" (www.diabeteshealth.com). Physicians in ancient times, like Aretaeus, recognized the symptoms of diabetes but were powerless to treat it effectively.

In the 17th century, a London physician Dr. Thomas Willis determined whether his patients had diabetes or not by sampling their urine. If it had a sweet taste he would diagnose them with DM-"honeyed" diabetes. This method of monitoring blood sugars went largely unchanged until the 20th century. Before the discovery of the insulin little could be done for patients suffering from diabetes. Low calorie diets prolonged their lives but left them weak and near starvation. But in 1921, doctors in Canada treated patients dying of diabetes with insulin and managed to drop high blood sugars to normal levels. Since then, medical breakthroughs have continued to prolong and ease the life of people with diabetes. In the 50s, it was discovered that there were two types of diabetes: "insulin sensitive" (type 1) and "insulin insensitive" (type 2).

Two thousand years have passed since Aretaeus spoke of diabetes as "the mysterious sickness". It has been a long and arduous process of discovery, as generations of physicians and scientists have added their collective knowledge to finding a cure. It was from this wealth of knowledge that the discovery of insulin emerged in a small laboratory in Canada. Since then, medical innovations have continued to make life easier for people with diabetes. In the 21st century, diabetes researchers continued to pave the road toward a cure. Today, it is unclear what shape the road will take; perhaps another dramatic discovery like insulin waits around the corner, or possibly researchers will have to be content with the slow grind of progress (Satley, 2008).

1.3 Epidemiology of Diabetes Mellitus

Frequency

There are 20.8 million children and adults in the United States or 7% of the population who have diabetes. While an estimated 14.6 million have been diagnosed with diabetes,

unfortunately, 6.2 million people (or nearly one-third) are unaware that they have the disease. It is estimated that 5-10% of Americans who are diagnosed with diabetes have T1D. Most of the rest have type 2. About 90% - 95% of all diabetes patients of Bangladesh belong to T2D (Frier et al., 2004).

Mortality/Morbidity

The morbidity and mortality associated with diabetes are related to the short- and long-term complications. Complications include hypoglycemia and hyperglycemia, increased risk of infections, micro-vascular complications (e.g. retinopathy, nephropathy), neuropathic complications and macro-vascular disease.

Sex

The incidence is essentially equal in women and men in all populations.

Age

T2D is becoming increasingly common because people are living longer and the prevalence of diabetes increases with age. It is also seen more frequently now than before in young people, in association with the rising prevalence of childhood obesity. Although T2D still occurs most commonly in adults aged 40 years or older, though the incidence of disease is increasing more rapidly in adolescents and young adults than in other age groups.

1.4 Pathophysiology of Diabetes Mellitus

An understanding of the pathophysiology of diabetes rests upon knowledge of the basics of carbohydrate metabolism and insulin action. Following the consumption of food, carbohydrates are broken down into glucose molecules in the gut. Glucose is absorbed into the bloodstream elevating blood glucose levels. This rise in glycemia stimulates the secretion of insulin from the β-cells of the pancreas (Figure 1.1). Insulin is needed by most cells to allow glucose entry. Insulin binds to specific cellular receptors and facilitates entry of glucose into the cell which uses the glucose for energy. The increased insulin secretion from the pancreas and the subsequent cellular utilization of glucose results in lowering of blood glucose levels. Lower glucose levels then result in decreased insulin secretion.

If insulin production and secretion are altered by disease, blood glucose dynamics will also change. If insulin production is decreased, glucose entry into cells will be inhibited, resulting in hyperglycaemia. The same effect will be seen if insulin is secreted from the pancreas but is not used properly by target cells. On the other hand, if insulin secretion is increased, blood glucose levels may become very low (hypoglycemia) as large amounts of glucose enter tissue cells and little remains in the bloodstream.

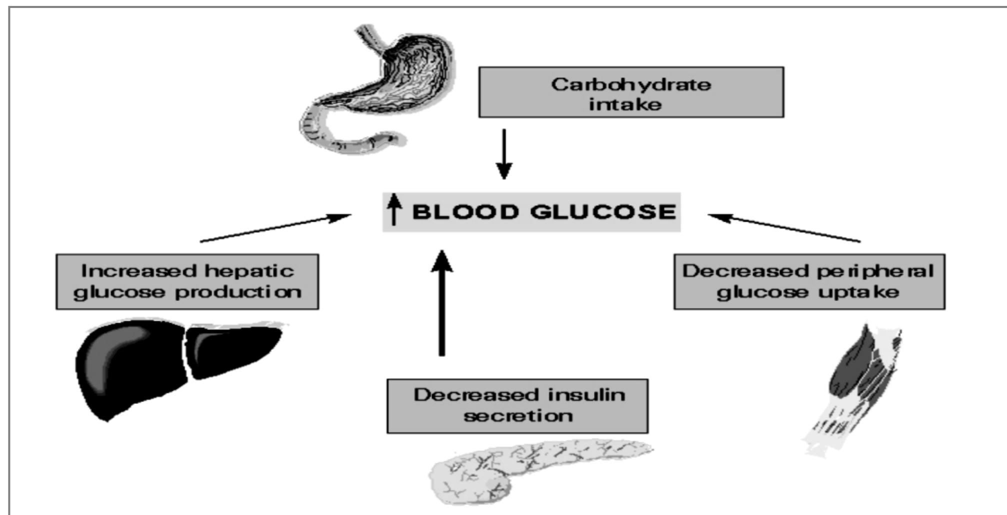


Figure 1.1: Simplified design of pathophysiology of T2DM (Pozzo, 2017).

Multiple hormones may affect glycemia. Insulin is the only hormone that lowers blood glucose levels. The counter-regulatory hormones such as glucagon, catecholamines, growth hormone, thyroid hormone and glucocorticoids all act to increase blood glucose levels in addition to their other effects (Mealey et al., 2006).

1.5 Complications of Diabetes Mellitus

Complications due to diabetes are a major cause of disability, reduced quality of life and death. Diabetes complications can affect various parts of the body manifesting in different ways for different people. Diabetes increases patient's risk for many serious health problems. In men, it is responsible for erectile dysfunction, low testosterone levels and emotional factors, such as depression, anxiety or stress that can interfere with sexual feelings. In women, diabetes can be especially hard. Even those who do not have diabetes,

pregnancy brings the risk of gestational diabetes. According to statistics from the American Diabetes Association (ADA), heart disease is the leading cause of death in women with diabetes. In addition, women with diabetes are afflicted by depression, their sexual health is at risk and eating disorders tend to occur more frequently.

However, diabetes can affect every part of the body, including the feet, the eyes and the skin. In fact, such problems are sometimes the first sign that a person has diabetes. Foot complications can get worse and lead to serious complications, such as neuropathy, skin changes, calluses as well as foot ulcers and poor circulation (Aalto, 1997).

1.6 Diagnosis of Diabetes Mellitus

The diagnosis of DM is easily established when a patient presents the classic symptoms of hyperglycaemia and has a random blood glucose value of 200 mg/dL (11.1 mmol/L) or higher, and confirmed on another occasion. The following tests are used for the basic diagnosis-

A fasting plasma glucose (FPG) test measures blood glucose in a person who has not eaten anything for at least 8 hours. This test is used to detect diabetes and prediabetes.

An oral glucose tolerance test (OGTT) measures blood glucose after a person fasts at least 8 hours and 2 hours after the person drinks a glucose-containing beverage. This test can be used to diagnose diabetes and prediabetes. The FPG test is the preferred test for diagnosing diabetes because of its convenience and low cost. However, it may miss some diabetes or prediabetes that can be found with the OGTT. The FPG test is most reliable when done in the morning. Research has shown that the OGTT is more sensitive than the FPG test for diagnosing prediabetes, but it is less convenient to administer.

A random plasma glucose test (RPGT), also called a casual plasma glucose test, measures blood glucose without regard to when the person being tested last ate. This test along with an assessment of symptoms is used to diagnose diabetes but not prediabetes. Test results indicating that a person has diabetes should be confirmed with a second test on a different day. The current WHO diagnostic criteria for diabetes should be maintained fasting plasma

glucose ≥ 7.0 mmol/L (126 mg/dL) or 2h plasma glucose ≥ 11.1 mmol/L (200 mg/dL) (World Health Organization, 1999).

1.7 Treatment of Diabetes Mellitus

Diabetes is now ranked as the sixth leading cause of death by disease in the U.S (Centers for Disease Control and Prevention, 2003). Its treatment as well as the management of diabetes-related complications remains a top priority for governments worldwide, since the economic burden in 2007 alone exceeded $174 billion (Dall et al., 2007).

1.7.1 Pharmacological treatments of Diabetes Mellitus

Old approaches to the treatment of this chronic progressive disease include diet modification and oral hypoglycemic medications which have proven inadequate, while insulin therapy only solves the problem temporarily. Even with the newest pharmaco-therapies, patients continue to develop macro- and micro-vascular complications. Diabetes is associated with increased cardiac- and stroke-related deaths, kidney failure, blindness and 60% of non-trauma lower-limb amputations (Centers for Disease Control and Prevention, 2003). Alternative treatments targeting different models of this disease require careful and responsible examination. Apart from insulin treatment, it is possible to gain diabetes control after gastrointestinal bypass surgeries.

A. Insulin based therapy

Diabetes, being one of the primary causes of increased cardiovascular morbidity and mortality in Western countries, constitutes a large burden to health care systems in terms of both direct and indirect costs. Therefore, efficient glucose control (attainment of normal HbA1C, prandial and postprandial glucose levels) is essential for the prevention of the life-threatening complications of this disease.

Insulin is a hormone that treats diabetes by controlling the amount of sugar (glucose) in the blood. When used as a medication, it is derived from either pork (porcine), beef (no longer available in the U.S.) or is genetically made to be identical to human insulin (Buysschaert, 2000). Patients with T1DM depend on external insulin (most commonly injected

subcutaneously) for their survival because the hormone is no longer produced internally. Patients with T2DM are insulin resistant, have relatively low insulin production, or both; certain patients with T2D may eventually require insulin if other medications fail to control blood glucose levels adequately.

There are many types of insulin used to treat diabetes. They are classified by how fast they start to work, when they reach their “peak” level of action (i.e. when the concentration of insulin in the blood is highest) and how long their effects last.

The types of insulin include-

i. Rapid-acting insulin, which starts working within a few minutes and lasts for a couple of hours.

ii. Regular- or short-acting insulin, which takes about 30 minutes to work and lasts for 3 to 6 hours.

iii. Intermediate-acting insulin, which takes 2 to 4 hours to work and its effects can last for up to 18 hours.

iv. Long-acting insulin, which takes 6 to 10 hours to reach the bloodstream, but it can keep working for an entire day (Tuomilehto, 2001).

Insulin for diabetes can be injected under the skin (subcutaneously) or into the vein (intravenously). Subcutaneous insulin injection continues to be the mainstay of therapy for all people with T1DM and the majority of individuals with T2DM. Insulin can be injected using a needle and syringe, a cartridge system, or prefilled pen systems. Insulin pumps are also available. The initial dose is calculated based on the patient’s weight and sensitivity to insulin, which varies from person to person. When given under the skin, insulin is typically taken so that two-thirds of the total daily dose is given in the morning and one-third of the total daily dose is given in the evening (Glasgow, 1999).

Complications of insulin therapy

Untreated hyperglycaemia can lead to long-term complications, including micro- and macrovascular complications. Tight glycaemic control with intensive insulin therapy has been suggested to reduce the risk of such complications in several diabetes populations; however, such an approach can also be associated with risks and challenges.

The major side effects of insulin taken for diabetes include low blood sugar (hypoglycemia), hypertrophy (enlargement of the area of the body that has received too many insulin injections) and rash at the site of injection or over the entire body (rare). The symptoms of the most common complication, i.e. low blood sugar, include extreme hunger, fatigue, irritability, cold sweats, trembling hands, intense anxiety and a general sense of confusion. They might be the signs of an insulin overdose, a potentially dangerous complication with diabetes which happens to many diabetic patients (Gkaliagkousi, 2007). Thankfully, most episodes related to insulin are avoidable if patients stick with a few simple rules.

Diabetic ketoacidosis (DKA) is another insulin complication which is the result of not taking enough insulin. In that case, excessive urination in response to high sugar causes severe dehydration. At the same time, without enough insulin to allow sugar absorption, the body's cells act as if they are starving. Without insulin, patients with T1D develop severely elevated blood sugar levels. This leads to increased urine glucose which in turn leads to excessive loss of fluid and electrolytes in the urine. Lack of insulin also causes the inability to store fat and protein along with breakdown of existing fat and protein stores. This deregulation results in the process of ketosis and the release of ketones into the blood. Ketones turn the blood acidic, a condition called DKA. Symptoms of DKA include nausea, vomiting and abdominal pain. Without prompt medical treatment, patients with DKA can rapidly go into shock, coma and even death (ibid).

DKA can be caused by infections, stress, or trauma, all of which may increase insulin requirements. In addition, missing doses of insulin is also an obvious risk factor for developing DKA. Urgent treatment of DKA involves the intravenous administration of fluid, electrolytes, and insulin, usually in a hospital intensive care unit. Dehydration can be

very severe, and it is not unusual to need to replace 6-7 liters of fluid when a person presents in DKA. Antibiotics are given for infections. With treatment, abnormal blood sugar levels, ketone production, acidosis and dehydration can be reversed rapidly and patients can recover remarkably well (Weymann et al., 2014).

Similar to DKA, hyperosmolar hyperglycemic nonketotic syndrome (HHNS) causes profound dehydration and can be life-threatening. It is an extremely serious complication that can lead to diabetic coma and even death in T2D. HHNS is much less common than DKA and tends to happen in older, obese patients with T2D (Buysschaert, 2000).

Once they occur, these insulin complications require hospitalization for treatment. The mainstays of treatment for both HHNS and DKA are the same: correction of fluid deficits, electrolyte imbalances and hyperglycaemia. In addition, it is particularly important in HHNS to identify and correct the underlying trigger condition. HHNS is often masked by the precipitating condition and comorbidities; it must be actively sought and the precipitating condition should be identified and treated.

In addition, HHNS has a high mortality rate. The fluid deficit is double than seen in DKA. The insulin therapy should be continued until the patient's mental status improves, hyperosmolality resolves and the target glucose level is reached.

B. Non-insulin based treatment

There is a relatively new class of drugs called incretinmimetics which mimic certain substances that can be found in the stomach and intestinal tract. These substances are normally released in response to food intake and signal the release of insulin from the pancreas. Since this reaction is reduced in people with T2D, incretinmimetics work to stimulate insulin release and help lower blood sugar. The doctor may recommend incretinmimetics if a patient has not been able to adequately control their blood sugar with other types of treatment. These medications are taken by injection, either once or twice a day.

For people with T2D, medications called DPP-4 inhibitors can be taken alone or in combination with other diabetes medications. DPP-4 inhibitors prevent the breakdown of incretin hormones. In turn, the incretins can help their body produce insulin to lower elevated blood sugar levels (Gkaliagkousi, 2007).

Oral Hypoglycemic Agents (OHA)

The term "oral hypoglycemic agent" can refer to any anti-diabetic medication. The following five categories will be presented in brief: Sulphonylureas and similar (secretagogues), Biguanides (sensitizers), Thiazolidindiones, Alpha glucosidase inhibitors, and Incretineanalogues/ agonists (Boulton, 2005).

As concerns the mode of action of the secretagogues like sulphonylureas, they block the ATP-sensitive K^+ channel and as a result they stimulate the insulin secretion. They are indicated to be the first choice once a patient is diagnosed with T2D. The side effects include hypoglycaemia and disulfiram reaction, while its interaction is the competition for protein binding/metabolism/secretion. The first-generation agents include tolbutamide (Orinase), acetohexamide (Dymelor), tolazamide (Tolinase), and chlorpropamide (Diabinese); the second-generation agents include glipizide (Glucotrol), glyburide (Diabeta, Micronase, Glynase), and gliclazide (Diamicron); the third-generation agents include glimepiride (Amaryl). The Meglitinides are short acting secretagogues, similar to sulphonylureas. They also block the ATP-sensitive K^+ and they open the Ca^{2+} channels, thus stimulating insulin secretion. Their side effects include weight gain and hypoglycaemia.

i. As concerns the mode of action of the sensitisers such as the Biguanides, they reduce the hepatic synthesis and the output of glucose, while they increase the insulin uptake in the skeletal muscle. They are actually antihyperglycaemic and not hypoglycaemic. They are indicated for T2DM and polycystic ovary syndrome (POS). They help reduce LDL cholesterol and triglyceride levels and they may help with the weight loss. Their side effects include lactate acidosis, GIT discomfort, diarrhea, renal toxicity, but they do not cause hypoglycemia.

ii. As concerns the mode of action of the glitazones such as the Thiazolidinediones, they are selective agonists of PPARγ receptors, thus activating the insulin-sensitive genes regulating the glucose and fat metabolism. As a result, they increase the insulin sensitivity in the periphereal tissue. Their side effects include hepatotoxicity (troglitazone).

iii. As concerns the mode of action of the inhibitors of the alpha-glucosidase or the alpha amylace, they reduce the intestinal absorption of starch, dextrins, and disaccharides and as a result they reduce the postprandial plasma glucose. They are indicated both for T1D and T2D, in combination with diet and insulin. Their side effects include malabsorption, flatulence and diarrhea.

iv. As concerns the mode of action of the peptide analogues such as the glucagon-like peptide-1 (GLP-1) agonists, they bind to a membrane GLP-1 receptor. They are metabolized by the dipeptidyl peptidase IV enzyme (DPP-IV). Their side effects include nausea, hypoglycemia (if given together with insulin secretagogue) and exenatide-acute pancreatitis. On the other hand, the mode of action of the GPP-IV inhibitors (gliptines) is that they increase the blood concentration of the incretin GLP-1 by inhibiting its degradation by DPP-IV. It has fewer side effects than other OHA, but the fact they constitute a new class of drugs should be taken into account (Gkaliagkousi, 2007).

1.7.2 Non-pharmacological treatment of Diabetes Mellitus

When it comes to non-pharmacological treatment of DM especially T2D lifestyle modification alone can prevent development of diabetes in impaired glucose tolerance patients. It can also be the sole therapeutic tool in early diabetes.

After being diagnosed with diabetes, a behavior and lifestyle modification is required. Health care providers should advice all diabetics not to initiate tobacco and emphasize stopping smoking in smokers as utmost priority for diabetic smokers, since it increases the risk of renal failure, visual impairment, foot ulcers, leg amputations and heart attacks in people with diabetes. The effects of stopping smoking in diabetes are substantial. The

incidence of micro- and macro-vascular complications was significantly increased in smokers compared to non-smokers (Buysschaert, 2000). As concerns, alcohol consumption of large amounts can cause hypoglycaemia and this can occur many hours after alcohol intake, particularly if no food has been consumed beforehand.

1.7.3 Metabolic surgery

Metabolic surgery is now emerging as an area dedicated to the establishment of surgical procedures specifically aimed at treating diabetes. In the early 1980s, surgeons realized that many patients with T2D who had undergone gastric bypass for the treatment of morbid obesity experienced a complete diabetes remission. This remission proved durable (Pories et al., 1995). Diabetes control and remission seems to be best obtained with procedures that include an intestinal bypass, as in gastric bypass or biliopancreatic diversion. With a 14-year follow-up, Pories et al. (1995) found an 83% resolution of T2D in 608 patients after gastric bypass. Remission is typically not seen until several months postoperatively, only once weight loss has occurred.

The mechanism of diabetes resolution after gastrointestinal bypass remains unclear but is apparently not related to weight loss alone. In most cases, remission is observed in the days to weeks after surgery before any substantial weight loss has occurred (Briatore et al., 2008). Furthermore, emerging evidence now shows that these effects may be achievable in the non-obese population as well. Clinical studies also show that the effect on diabetes after gastric bypass procedures does not depend only on the amount of weight loss (DePaula et al., 2008).

The surgical treatment of diabetes may be the answer to the global health crisis of the next generation. However, as with all other surgical procedures, the benefits of surgery must be weighed against the potential risks. In other words, one must consider the possible complications and mortality of surgery versus the probable remission of diabetes and decrease in lifelong diabetes-related morbidity and mortality. Contrary to commonly held misperceptions, bariatric surgery has a strikingly safe operative profile and associated low mortality (Rubino et al., 2009). The benefits of diabetes resolution accomplished by

surgery are significant. Diabetes-related mortality after Roux-en-Y gastric bypass has been followed over a period of 7 years and decreased 92% compared with controls (Adams et al., 2007). Another benefit of the surgery is the general improvement in metabolic syndrome, which contributes to a decrease in cardiovascular risk factors. Studies have shown a significant improvement in all components of the metabolic syndrome (T2D, hypertension, increased fasting glucose and triglycerides, decreased HDL, and abdominal obesity) and an overall resolution of 95.6% at 1 year (Rossi et al., 2008).

Surgery seems to provide an additional weapon against diabetes. Despite the compelling outcome data, the decision to operate should be made based on a risk factor assessment for each patient. Surgery is by design an invasive treatment modality and carries risks related to both anesthesia and the procedure itself. The potential benefits of metabolic surgery are in fact enormous. However, its implementation requires a rethinking of diabetes treatments goals and strategies. In the meantime, investigation into the pathophysiological basis of diabetes continues, with the hope of discovering the optimal therapeutic targets and best-suited interventions.

1.7.4 Diet and Diabetes Mellitus

The major environmental factors that lead to T2D are sedentary lifestyle and over nutrition leading to obesity (Harris, 1991). Sedentary lifestyle is more common in urbanized societies.

Dietary advice is essential upon diagnosis of diabetes. Normal advice includes-

- Reducing intake of fatty foods.
- Eating mainly vegetables, fruit, cereal, rice and pasta (using whole meal products where possible).
- Eating only small amounts of refined sugar (jam, sweets etc.).
- Eating at regular intervals.

- Carrying glucose tablets, sweets or products in case of hypoglycaemia.
- Exercising regularly; not only does it help reduce hyperglycaemia, but it also reduces insulin resistance by reducing obesity.

Most cases are preventable with healthy lifestyle changes and some can even be reversed. Taking steps to prevent and control diabetes doesn't mean living in deprivation. While eating right is important, patients don't have to give up sweets entirely or resign themselves to a lifetime of "health food".

Carbohydrates have a big impact on blood sugar levels more so than fats and proteins. In general, patients should limit highly refined carbohydrates like white bread, pasta, and rice, as well as soda, candy and snack foods. Focus instead on high-fiber complex carbohydrates-also known as slow-release carbs. Slow-release carbs help keep blood sugar levels even because they are digested more slowly, thus preventing the body from producing too much insulin. They also provide lasting energy and help people stay full longer (Gross, 2005).

1.7.5 Exercise and Diabetes Mellitus

Physical activity reduces the risk of developing T2D by 30-50% and risk reductions are observed with as little as 30 minutes of moderate exercise per day (Gkaliagkousi, 2007). Regular exercise improves glycaemic control in all forms of diabetes. Insulin resistance is the major cause of hypoglycemia in T2D and physical exercise is the best way to reduce insulin resistance (Goodpaster et al., 2010). Physical activity improves insulin sensitivity in many ways. Fat accumulation in the liver is the main cause of insulin resistance in obesity. Exercise can reduce the free fatty acid load to liver and thereby reduce hepatic insulin resistance (Haus et al., 2010). Exercise recommended is moderate exercise for 30 minutes a day (Tuomilehto et al., 2001) or moderate physical activity like brisk walking at least 150 minutes per week (Diabetes Prevention Programme research group in *NEJM* 2002). Putative protective mechanisms include reduction of body weight; reduction of insulin resistance, and thereby the associated consequences of the metabolic syndrome,

including hypertension, dyslipidaemia and inflammation; and enhancement of endothelial function (Gkaliagkousi, 2007).

There are further benefits from staying active apart from losing weight and keeping fit. According to the American Diabetes Association (ADA), physical activity improves glucose management, lowers blood pressure, improves blood fats, as well as reduces the amount of insulin or diabetic pills after losing weight. It also helps keep off the weight a person loses and lowers the risk for other health problems. Physically active people will soon discover that they gain more energy and get better sleep as a result of action, which also reduces stress, anxiety and depression. Physical activities build stronger bones and muscles and helps people of all ages stay more flexible (American Diabetes Association: Standards of medical care in diabetes, 2007).

1.7.6 Biological drugs for treatment of Diabetes Mellitus

Biological therapy is treatment designed to stimulate or restore the ability of the body's immune system to fight infection and disease. Biological therapy is also called biotherapy or immunotherapy. Biological drugs are defined as medicines the active substance of which comes from a biological source.

These drugs are very different from normal prescription drugs and are developed through advanced technology called “genetic modification”. Most of the biological drugs today are monoclonal antibodies (mABs). They can fight disease in the same way normal antibodies do, but they have been specially changed to find the specific area that needs treatment and to treat a specific disease with fewer side effects (Haus et al., 2010).

In T1D, insulin-producing pancreatic β-cells are attacked and destroyed by the immune system. Although man-made insulin is life-saving, it is not a cure and it cannot prevent long-term complications. In addition, most T1D patients would do almost anything to achieve release from the burden of daily glucose monitoring and insulin injection. Despite the formation of very large and promising clinical trials, a means to prevent and cure the disease in humans remains elusive. This has led to an increasing interest in the possibility

of using T cells with regulatory properties (Treg cells) as a biological therapy to preserve and restore tolerance to self-antigens (ibid).

T1D accounts for 5% of the diabetic cases. Patients can no longer produce their own insulin either as a result of the destruction of the patient's β-islet cells of the pancreas by autoreactive T-cells or due to the neutralizing effect of auto-antibodies directed against insulin. The only treatment option today is insulin replacement.

An ActoBiotic developed by ActoGeniX is capable of delivering pro-insulin and IL10 in the gut, showing a restoration of antigen-specific, long-term tolerance and reversal of diabetes in NOD mice, combined with low-dose anti-CD3 (reference). Mechanistically, this intervention approach increases local regulatory T-cell frequencies which proliferate in the pancreatic islets and suppress the auto-immune reaction in an antigen-specific way (ibid). mABs, like Otelixizumab and Rituximab can be used in the treatment of T1D.

Otelixizumab

Otelixizumab, also known as TRX4, is a humanized anti-CD3 monoclonal antibody, which has been developed by Tolerx, Inc. as a treatment for autoimmune disorders, including T1D. Otelixizumab targets CD3, a T lymphocyte receptor involved in normal cell signaling. It blocks the function of effector T cells, which mistakenly attack and destroy insulin-producing beta cells, while stimulating regulatory T cells, which are understood to protect against effector T cell damage, thus preserving the β- cell's normal ability to make insulin.

In 2011, Otelixizumab failed in a pivotal phase 3 study, leaving safety and efficacy issues of biological drugs open to speculation. Otelixizumab shows great promise but leaves room for improvement. Results of ongoing trials will help define its role in the prevention of T1DM (Goodpaster, 2010). Rituximab is a mABs against the protein CD20, which is primarily found on the surface of β-cells. It is used as a treatment to some autoimmune disorders, including T1D. T1D is believed to be due to the autoimmune destruction of β-cells by T lymphocytes. It has been reported that a single course of Rituximab can attenuate C-peptide loss over the first year of disease (Daneman, 2006).

Biological drugs have been proven to help not only with the treatment of diabetes itself but also with the complications from which the patients suffer. In particular, they are used as a cure to diabetic foot ulcers and diabetic kidney disease. Therefore, patients can fight not only diabetes but other annoying issues as well. Prompt biological therapy has been proven to speed up diabetic foot ulcer healing.

1.8 Prevention and management of diabetes complications

Diabetes is a group of chronic diseases characterized by hyperglycaemia. Modern medical care uses a vast array of lifestyle and pharmaceutical interventions aimed at preventing and controlling hyperglycaemia. In addition to ensuring the adequate delivery of glucose to the tissues of the body, treatment of diabetes attempts to decrease the likelihood that the tissues of the body are harmed by hyperglycaemia. The importance of protecting the body from hyperglycaemia cannot be overstated; the direct and indirect effects on the human vascular tree are the major source of morbidity and mortality in both T1D and T2D.

Diabetes complications are divided into two major categories-

a. Acute complications such as hypoglycaemia and comas resulting either form DKA or HHNS (as stated above) and

b. Chronic complications, either micro-vascular (diabetic retinopathy, nephropathy, neuropathy) or macro-vascular (coronary artery disease, peripheral arterial disease and stroke)

1.8.1 Micro-vascular complications

The most serious micro-vascular diabetes complications are the eye complications. Diabetic patients are strongly advised to have an annual ophthalmic exam. Diabetic retinopathy (DR) is the leading cause of blindness in the working population of the Western world. The risk of developing DR or other micro-vascular complications of diabetes depends on both the duration and the severity of hyperglycaemia (Almdal, 2004). DR is generally classified as either background or proliferative. It is important to have a general understanding of the features of each to interpret eye examination reports and

advise patients of disease progression and prognosis. Background retinopathy includes such features as small hemorrhages in the middle layers of the retina. They clinically appear as "dots" and therefore are frequently referred to as "dot hemorrhages." Proliferative retinopathy is characterized by the formation of new blood vessels on the surface of the retina and can lead to vitreous hemorrhage (Watkins, 2003).

Diabetic nephropathy (DNp) is the leading cause of renal failure in the United States. About 20% to 30% of the patients with diabetes develop evidence of nephropathy. Initial treatment of DNp, as of other complications of diabetes, is prevention. Like other micro-vascular complications of diabetes, there are strong associations between glucose control and the risk of developing DNp. Patients should be treated to the lowest safe glucose level that can be obtained to prevent or control DNp (Gross et al., 2005). In addition to aggressive treatment of elevated blood glucose, patients with DNp benefit from treatment with antihypertensive drugs. Renin-angiotensin system blockade has additional benefits beyond the simple blood pressure-lowering effect in patients with DNp (Gross et al., 2005).

Diabetic neuropathy (DNu) is recognized by the American Diabetes Association (ADA) as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" (Nathan et al., 2007). As with other micro-vascular complications, risk of developing DNu is proportional to both the magnitude and duration of hyperglycaemia, and some individuals may possess genetic attributes that affect their predisposition to developing such complications.

The precise nature of injury to the peripheral nerves from hyperglycaemia is not known but likely is related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress. Peripheral neuropathy in diabetes may manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations occur after foot ulceration or injury, which can result from DNu (Boulton et al., 2005).

Diabetic autonomic neuropathy also causes significant morbidity and even mortality in patients with diabetes. Neurological dysfunction may occur in most organ systems and can by manifest by gastroparesis, constipation, diarrhea, anhidrosis, bladder dysfunction, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and even sudden cardiac death (Boulton et al., 2005). Patients may be asymptomatic or complain of numbness, burning, tingling, and “electrical” pain. While the feet are mostly affected, the hands are seldom affected.

1.8.2 Macro-vascular complications

The central pathological mechanism in macro-vascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system (Lehto, 1996).

Diabetes increases the risk that an individual will develop cardiovascular disease (CVD). Although the precise mechanisms through which diabetes increases the likelihood of atherosclerotic plaque formation are not completely defined, the association between the two is profound (Laing et al., 2003). CVD is a major complication and the leading cause of premature death among diabetic patients (Merz et al., 2002). Diabetic patients have a 2 to 6 times higher risk for developing complications such as ischemic heart disease, CVD and peripheral vascular disease than the general population. Among macro-vascular diabetes complications, coronary heart disease has been associated with diabetes in numerous studies beginning with the Framingham study (Kannel et al., 1979).

The major cardiovascular risk factors in the non-diabetic population (smoking, hypertension and hyperlipidemia) also operate in diabetes, but the risks are enhanced in the presence of diabetes. Overall life expectancy in diabetic patients is 7 to 10 years shorter than non-diabetic people.

T2D typically occurs in the setting of the metabolic syndrome, which also includes abdominal obesity, hypertension, hyperlipidemia, and increased coagulability. These other factors can also act to promote CVD. Even in this setting of multiple risk factors, T2D acts

as an independent risk factor for the development of ischemic disease, stroke, and death (Almdal et al., 2004), Diabetes is also a strong independent predictor of risk of stroke and cerebrovascular disease, as in coronary artery disease (Lehto et al., 1996).The increased risk of CVD has led to more aggressive treatment of these conditions to achieve primary or secondary prevention of coronary heart disease before it occurs. Studies in T1D have shown that intensive diabetes control is associated with a lower resting heart rate and that patients with higher degrees of hyperglycaemia tend to have a higher heart rate, which is associated with higher risk of CVD (Paterson et al., 2007).

Once clinical macro-vascular disease develops in diabetic patients they have a poorer prognosis for survival than normoglycaemic patients with macro-vascular disease. The protective effects females have for the development of vascular disease are lost in diabetic females. In addition, the combination of hypertension and diabetes is a serious situation, posing increased predisposition to cardiovascular morbidity and mortality. There is no doubt that hypertension occurs more commonly in diabetic patients, and confer a greater prospect of development of complications; it should therefore be taken as seriously as glycaemic control when planning treatment strategies.

Hyperlipidemia can occur as a result of poorly controlled diabetes, or may occur as an independent risk factor for macro-vascular disease. About 25% of patients attending a diabetes clinic will have elevated lipid levels (Jacobson, 1985).

1.8.3 Infections

Diabetic patients are also in greater risk of infections than healthy individuals. The association between diabetes and increased susceptibility to infection in general is not supported by strong evidence. However, many specific infections are more common in diabetic patients and some occur almost exclusively in them. Other infections occur with increased severity and are associated with an increased risk of complications.

Several aspects of immunity are altered in patients with diabetes. There is evidence that improving glycaemic control improves immune function. Fungal cystitis, rhino-cerebral mucormycosis and community-acquired pneumonia are among the most common infections the diabetic patients suffer from (Gu, 1998).

1.8.4 Recommendations for managing the complications of diabetes mellitus

a. Blood pressure should be measured routinely. Goal blood pressure is <130/80 mmHg. Patients with a blood pressure ≥140/90 mmHg should be treated with drug therapy in addition to diet and lifestyle modification.

b. Patients with a blood pressure of 130-139/80-89 mmHg may attempt a trial of lifestyle and behavioral therapy for three months and then receive pharmacological therapy if their goal blood pressure is not achieved.

c. Lipid testing should be performed in patients with diabetes at least annually. Lipid goals for adults with diabetes should be an LDL <1000 mmol/l (or <700mmol/l in patients with overt CVD), HDL >500mmol/l, and fasting triglycerides <1500mmol/l.

d. All patients with diabetes should be encouraged to limit consumption of saturated fat, trans fat, and cholesterol.

e. Patients with type I diabetes should receive a comprehensive eye examination and dilation within 3-5 years after the onset of diabetes. Patients with T2D should undergo such screening at the time of diagnosis.

f. Patients should strive for optimal glucose and blood pressure control to decrease the likelihood of developing diabetic retinopathy or experiencing progression of retinopathy (Paterson et al., 2007).

g. All patients with diabetes should undergo screening for distal symmetric polyneuropathy at the time of diagnosis and yearly thereafter.

h. Patients who experience peripheral neuropathy should begin appropriate foot self-care, including wearing special footwear to decrease their risk of ulceration.

i. In addition to the above pharmacological recommendations, patients with diabetes should be encouraged to not begin smoking or to stop smoking to decrease their risk of CVD and benefit their health in other ways.

1.9 Classification of Diabetes Mellitus

There are three main forms of this metabolic disease and their prevalence is increasing worldwide.

Type 1 diabetes (T1D), also called insulin dependent diabetes mellitus, is characterized by little or no insulin production and weight loss, and accounts for between 5 and 10 percent of all diabetes mellitus (Rich SS et al., 2009).

Type 2 diabetes (T2D), also called non-insulin dependent diabetes mellitus, is caused by cells becoming resistant to insulin signaling and accounts for 85 to 90 percent of diabetes worldwide (Zimmet et al., 1982). Diet, obesity, and reduced physical activity are a few of the important factors that are thought to contribute to the development of T2D.

The third type, gestational diabetes mellitus (GDM), is a temporary condition that occurs during pregnancy. It is defined as "carbohydrate intolerance of variable severity with onset or first recognition during the present pregnancy" (Russell et al., 2007). Several studies have shown that gestational diabetes mellitus is associated with an increased risk of obstetric complications such as preeclampsia (development of new hypertension and proteinuria), cesarean delivery and fetal macrosomia (abnormally large size of the body) (Rosenberg et al., 2005; Xiong et al., 2001). An improved pregnancy outcome can result from the early detection of this condition and an appropriate treatment (Crowther et al., 2005).

1.9.1 Type 1 diabetes mellitus (T1DM)

T1D results from autoimmune destruction of the pancreatic β-cells. Markers of immune destruction of the β-cell are present at the time of diagnosis in 90% of individuals and include antibodies to the islet cell (ICAs), to glutamic acid decarboxylase (GAD) and to insulin (lAAs). While this form of diabetes usually occurs in children and adolescents, it can occur at any age. Younger individuals typically have a rapid rate of β-cell destruction and present with ketoacidosis, while adults often maintain sufficient insulin secretion to prevent ketoacidosis for many years. The more indolent adult-onset variety has been referred to as latent autoimmune diabetes in adults (LADA). Eventually, all T1DM patients will require insulin therapy to maintain normglycemia .

T1DM is a chronic state of insulin deficiency which results from destruction of beta cells by the immune system. The long term microvascular and macrovascular complications can be devastating. Since the discovery of insulin almost 100 years ago new medical therapies have improved the long-term survival for people with T1DM. Each year we come closer to discovering a cure but much work still needs to be done to eliminate this disease.

Prior to the discovery of insulin, a diagnosis of diabetes was fatal within a few weeks to months due to insulin deficiency. With the discovery of insulin people with T1DM were able to live productive lives for many decades. However in 2017, the life expectancy of people with T1D is still approximately 12 years less on average than the rest of the general population. The diabetes control and complications trial showed us that intensive control of T1D leads to a decrease in microvascular complications such as retinopathy, nephropathy, and neuropathy. The epidemiology of diabetes interventions and complications study showed that intensive blood glucose control reduces the risk of cardiovascular disease. The age adjusted relative risk for cardiovascular disease in people with T1D is still 10 times that of the general population. The increased mortality and the burden of long-term diabetes care indicate that there is still much we need to learn about T1D prevention, treatment and finding a true cure for this disease.

Etiology of type 1 diabetes mellitus

T cell mediated autoimmune destruction of pancreatic beta cells is thought to be the final pathway in the development of type 1 diabetes (T1DM). Multiple beta cell autoantibodies are frequently present in patients with T1DM. Antigens for these antibodies include insulin, glutamic acid decarboxylase (GAD-65), islet antigen 2, and zinc-transporter 2. The role of these antibodies in causing beta cell destruction is not completely understood. How this autoimmune process is triggered is not known, although both genetic and environmental factors are thought to be necessary. These holes in our knowledge are critical impairments in our ability to prevent T1DM. Figure 1.2 shows a diagram of the proposed mechanisms behind the development of T1DM.

The major genetic determinants of T1D are alleles at the HLA-DRB1 and DQB1 loci. DQA1*0501 and DQB1*0302 confer very high risk for T1D. Kingery et al. have suggested a role for complement component 4 (C4) copy number variation in the development of the disease. This is interesting since the C4 gene is closely associated to the HLA locus. Polymorphisms in multiple other genes including the insulin gene have also been found to play a role, although their relative contribution is small.

The exact environmental factors involved in T1D initiation are far less known. One plausible hypothesis is that viral infections trigger beta cell autoimmunity in genetically susceptible individuals. Several viruses have been associated with T1D, including enteroviruses such as coxsackievirus B. Enterovirus infections are more frequent in siblings who develop type 1 diabetes compared with siblings without diabetes. Enterovirus antibodies are elevated in pregnant mothers whose children develop diabetes, particularly in cases diagnosed before 3 years of age. Coxsackievirus B is one of the most common enteroviral strains found in people with pre-diabetes and diabetes, and enteroviral RNA has been found in samples taken from children at the onset of T1D.

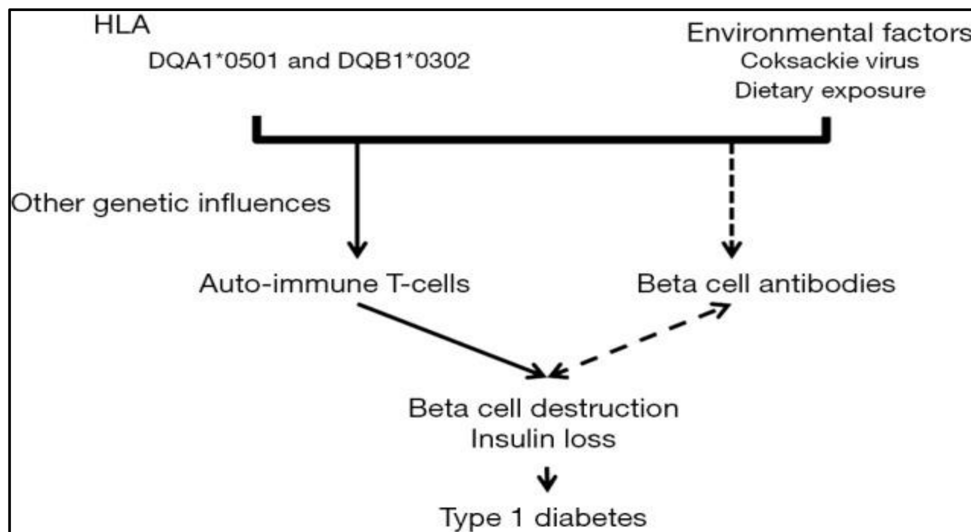


Figure 1.2: Proposed etiology of type 1 diabetes. Environmental factors in genetically susceptible individual trigger auto-immune T-cells that lead to beta cell destruction and insulin loss. HLA confers most of the genetic risk although genetics risk factors have been identified. It is unknown how humoral immunity and beta cell antibodies relate to beta-cell destruction. They may play an active role or may simply be a marker of ongoing destruction (Copenhaver & Hoffman, 2017).

Although there have been viruses found in pancreatic beta cells, viruses may not necessarily be the trigger for T1D and could possibly be protective since some countries with different sanitary standards and lower socioeconomic status tend to have more infections but a lower prevalence of T1D. The role of the complement system and enteroviruses in the development of T1D was recently explored by Abdel-Latif *et al.* who compared T1D children who were enterovirus positive children with those children who had T1D but were enterovirus negative and looked at autoantibodies, cytokines, complement activation products, and anti-coxsackievirus immunoglobulin IgG. The higher serum levels of complement components C3d and sC5b-9 indicated increased complement activity in diabetes enterovirus positive children versus diabetic enterovirus negative children. The enterovirus negative children with diabetes did not show any significant differences in complement levels compared to healthy controls.

1.9.2 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is the least common form of diabetes. GDM is defined as an impairment in carbohydrate metabolism that begins or first manifests during pregnancy. One reason diabetes occurs during pregnancy is that some hormones; for example, human placental lactogen, that have anti-insulin properties are produced by the placenta. As the placenta grows, the production of these hormones increases leading to more carbohydrate intolerance and insulin resistance. Studies have shown that patients with GDM are at higher risk for excessive weight gain and preeclampsia (Perkins et al., 2007). Moreover, infants born to mothers with GDM are at higher risk of developing macrosomia, hypoglycemia and subsequent obesity and T2D. Macrosomia results from the fetus being exposed to too much insulin. This is because the fetus is exposed to the maternal hyperglycemia, but the maternal insulin does not cross the placenta. The maternal and fetal complications associated with GDM can be reduced by recognizing and treating GDM (Perkins et al., 2007). Many factors have been reported to contribute to the susceptibility of developing GDM. These include race, age, parity, family history of diabetes, pre-pregnancy weight, postpartum obesity, and weight gain, which have been reported to play a role in the development of GDM (Kim et al., 2002; Linne et al., 2002). Other suspected risk factors include smoking, physical inactivity, diet, and drugs that adversely affect glucose metabolism (Dornhorst et al., 1998).

Women who have had GDM have a 35% to 60% chance of developing diabetes in the next 10–20 years. GDM is also associated with an increased risk of developing subsequent T2D (Aberg et al., 2002). This is supported by Perkins and colleagues study that having a history of GDM puts the mother at risk for developing T2D in the future or recurrent GDM (Perkins et al., 2007). There is also a relationship between GDM and cardiovascular disease. Carr et al. (Carr et al., 2006) conducted a study examining whether GDM increases the risk of cardiovascular disease in women with a family history of T2D. Researchers found that these women not only had a higher prevalence of cardiovascular disease, but also were more likely to have experienced the disease events at a younger age (Carr et al., 2006).

1.9.3 Type 2 diabetes mellitus (T2DM)

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. T2D is characterized by insulin resistance or relative insulin deficiency. It is the most common form and comprises of 90% of people with diabetes around the world. The prevalence of T2D rates continue to increase with increasing number of patients at risk of serious diabetes-related complications. T2D increases the risk of a myocardial infarction two times and the risk of suffering a stroke two to four times. It is also a leading cause of blindness, limb amputation and kidney failure (Federation, 2006). Although trials of secondary prevention after myocardial infarction show as good or better short term effect of interventions in patients with diabetes as in patients without, patients with diabetes have not had a similar reduction in longer-term case fatality rates of cardiovascular disease (CVD) (Cubbon et al., 2007). Population based studies of CVD risk factor trends among subjects with and without diabetes show differing trend in disfavor of those with diabetes (Preis et al., 2009). Studies of adherence to guidelines for CVD prevention targets in patients with diabetes in general practice have shown that only 13% reach all the targets (Tuomilehto et al., 2001). Previous studies have found appropriate lifestyle intervention and/or drug treatments are effective in delaying or preventing both diabetes and its complications (Ramachandran et al., 2008). Accordingly, simple, sensitive and acceptable tools for identification of subjects at risk are warranted.

1.9.3.1 Epidemiology of T2DM

The world prevalence of diabetes in 2010 among adults aged 20-79 years is estimated to 6.4%, affecting 285 million adults (Shaw et al., 2010). Between 2010 and 2030, there is an expected 70% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries (Shaw et al. 2010). Each year more than 231,000 people in the United State and more than 3.96 million people worldwide die from diabetes and its complications (Preis et al., 2009).

1.9.3.2 Risk factors for T2DM

Many studies have elaborated the associations between several risk factors and the risk of T2D. Body mass index (BMI), lipids, hypertension, smoking, physical inactivity, low education, dietary patterns, family history and recently also specific genes are the most frequently documented risk factors for T2D (Lyssenko et al., 2008).

BMI

Many longitudinal studies have reported that increased BMI is a strong risk factor for T2D (Meisinger et al., 2002; Knowler et al., 1991; Kumari et al., 2004; Njolstad et al., 1998; Almdal et al., 2008). A strong positive association between obesity and T2D is found both in men (Knowler et al., 1991; Almdal et al., 2008; Manson et al., 1992; Skarfors et al., 1991). Obesity is associated with increased risk of developing insulin resistance and T2D. In obese individuals adipose tissue releases increased amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other factors involved in the development of insulin resistance. When insulin resistance is accompanied by dysfunction of the beta cells, the following fall in insulin secretion results in failure to control blood glucose level leading to T2D. Many genes interact with the environment leading to obesity and in some also to diabetes. Many genes have been shown to be involved in determining the whole range of BMI in a population, with each gene only explaining a few hundred grams difference in body weight (Hebebrand et al., 2009). Genes responsible for obesity and insulin resistance interact with environmental factors such as increased fat/ calorie intake and decreased physical activity resulting in the development of obesity and insulin resistance followed ultimately by the development of T2D (Kahn et al., 2006; O'Rahilly et al., 2006).

Lipids

Unfavorable blood lipids have been reported as a risk factor for T2D by several prospective studies (Meisinger et al., 2002; Haffner et al., 1997; Knowler et al., 1991; Njolstad et al., 1998; Almdal et al., 2008; Jacobsen et al., 2002). An inverse relationship between HDL cholesterol and risk of T2D have been documented in several of studies (Meisinger et al., 2002; Haffner et al., 1997; Almdal et al., 2008; Jacobsen et al., 2002). Some prospective studies found low HDL cholesterol to be a stronger risk factor for T2D in women only (Almdal et al., 2008; Fagot-Campagna et al., 1997). High plasma triglycerides and low plasma HDL cholesterol levels are both seen in the insulin resistance syndrome, which is a prediabetic state (Taskinen et al., 2003; Reaven et al., 1988), suggesting that nonfasting triglycerides and HDL cholesterol levels reflect the degree of insulin resistance. Apart from triglycerides, all lipids have been shown to convey diabetes risk independently of BMI, but how they interact has been little studied.

Hypertension

Previous prospective studies have shown that hypertension progression is an independent predictor of T2D (Kumari et al., 2004; Conen et al., 2007; Gress et al., 2000; Movahed et al., 2010). Several possible factors are likely causes of the association between type 2 diabetes and hypertension. Endothelial dysfunction could be one of the common pathophysiological pathways explaining the strong association between blood pressure and incident T2D. Studies have shown that markers of endothelial dysfunction are associated with new-onset of diabetes (Meigs et al., 2004; Meigs et al., 2006), and endothelial dysfunction is closely related to blood pressure and hypertension (Gokce et al., 2001). In addition evidence from cross sectional and cohort studies suggests a strong relation between blood pressure and BMI and risk of T2D (Must et al. 1999; Wild et al., 2006; Czernichow et al., 2002; Wilsgaard et al., 2000).

Smoking

Several prospective studies reported that current smoking is a risk factor for developing T2D (Hu et al., 2001; Hur et al., 2007; Yeh et al., 2010; Manson et al., 2000; Sairenchi et al., 2004). Recently, a meta- analysis including 25 prospective studies showed that current

smoking was associated with a 44% increased risk of diabetes (Willi et al., 2007). Smoking leads to insulin resistance and inadequate compensatory insulin secretion response (Attvall et al., 1993; Facchini et al., 1992; Janzon et al., 1983). Some studies suggest that heavy smokers with evidence of increased systemic inflammation who gain substantial in weight after quitting, are at high risk of developing T2D (Yeh et al., 2010; Duncan et al., 2003). However over longer follow up, smoking cessation is associated with a reduction in risk of developing T2D (Wannamethee et al., 2001).

Physical inactivity

Longitudinal studies have found physical inactivity to be a strong risk factor for T2D (Almdal et al., 2008; Fretts et al., 2009; Gimeno et al., 2009; Villegas et al., 2006; Jeon et al., 2007). Prolonged television watching as a surrogate marker of sedentary lifestyle was reported to be positively associated with diabetes risk in both men and women (Hu et al., 2003; Hu et al., 2001; Krishnan et al., 2008). Moderate and vigorous physical activity was associated with a lower risk of T2D (Manson et al., 1992; Fretts et al., 2009; Weinstein et al., 2004). Evidence from clinical trials which included physical activity as an integral part of life style interventions suggested that onset of T2D can be prevented or delayed as a result of successful lifestyle interventions that included physical activity as a part of these interventions (Tuomilehto et al., 2001; Knowler et al., 2002; Ramachandran et al., 2006; Pan et al., 1997). Physical activity plays an important role in delaying or prevention of development of T2D in those at risk both directly by improving insulin sensitivity and reducing insulin resistance, and indirectly by beneficial changes in body mass and body composition (Hamma et al., 2006; Boule et al., 2001; Kay et al., 2006).

Low education

Previous prospective studies have examined the association between educational attainment and the incidence of diabetes and found that low education is significant predictor of T2D (Valdes et al., 2007; Maty et al., 2005; Leonetti et al., 1992). In a cross sectional study of National Population Health Survey found that people with less than high school diploma were almost twice as likely to report having diabetes as those with a bachelor degree or more (Tang et al., 2003). Another cross sectional study from the

National Health Interview Survey found that women with low education had a higher prevalence of diabetes than the better educated. A recent cross sectional study found that T2D risk was higher in the least educated who were obese and inactive compared to the more educated (Dasgupta et al., 2010). These studies suggest that educational attainment promote an interest in own health and acquisition of knowledge that strongly influence people's ability to reduce risk by successfully adopting a healthier life style.

Dietary pattern

An important life style factor associated with the development of T2D is dietary habits. Positive associations have been reported between the risk of T2D and different patterns of food intake (Liese et al., 2008; Sun et al., 2010; van Dam et al., 2002; Schulze et al., 2004). Higher dietary glycemic index has been consistently associated with elevated risk of T2D in prospective cohort studies (Schulze et al., 2004; Villegas et al., 2007). A prospective study found that regular consumption of white rice is associated with an increased risk of T2D whereas replacement of white rice by brown rice or other whole grains was associated with a lower risk (Sun et al., 2010). Concluded that a higher intake of polyunsaturated fat and long- chain n.3 fatty acid is beneficial, where as higher intake of saturated fat and trans fat adversely affects glucose metabolism and insulin resistance (Hu et al., 2001). Another prospective study found higher consumption of butter, potatoes and whole milk to be associated with increased risk of type 2 diabetes. Higher consumption of fruits and vegetable was associated with reduced risk of T2D (Montonen et al., 2005). The possible mechanisms suggested are that insoluble fiber intake was consistently associated with improved insulin sensitivity and decreases risk of T2D (Salmeron et al., 1997; Meyer et al., 2000). Furthermore large observational studies have suggested an association between low vitamin D status or low vitamin D intake and increased incidence of T2D (Knekt et al., 2008; Pittas et al., 2006). The suggested mechanisms are that vitamin D deficiency may contribute to beta cell dysfunction, insulin resistance and inflammation that may result in T2D. The effect of dietary habits has in all these studies been shown to be independent of BMI change.

Genetics

Several studies have found that genetic components play an important role in pathogenesis of T2D (Amini et al., 2007; Meigs et al., 2000; Harrison et al., 2003; Diamond et al., 2003). Several prospective studies and cross sectional studies have reported that positive family history among first degree relatives confers an increased risk of T2D and the risk is greater when both parents are affected (Amini et al., 2007; Meigs et al., 2000; Ma et al., 2008; Bjornholt et al., 2000). Also, diabetes prevalence varies substantially among different ethnic groups, and this observation of substantial variation of disease prevalence across ethnic groups that share a similar environment, supports the idea that genetic factors contribute to disease predisposition (Das et al., 2006). Data from multiple laboratories support that genetic factors predispose to development of T2D by reducing insulin sensitivity and insulin secretion which deteriorate in parallel in most human T2D cases (Das et al., 2006; Elbein et al., 2000; Gerich et al., 1998). Recent studies have identified variants in 11 genes (*TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1,* and *HHEX*) to be significantly associated with the risk of type 2 diabetes independently of other clinical risk factors and variants in 8 of these genes were associated with impaired beta-cell function. Among these genes expressed in pancreatic cells and involved in impairment of insulin secretion, the transcription factors 7-like 2 (*TCF7L2*), is the locus with the highest risk of T2D (HR 1.5) (Lyssenko et al., 2008; Cauchi et al., 2008; Lyssenko, 2008; Prokopenko et al., 20008).

So far genetic information is of interest for research purposes only.

1.9.3.3 Prevention of T2DM

Before people develop T2D, they almost always have "prediabetes"–blood glucose levels that are higher than normal but not yet high enough to be diagnosed as diabetes. Prediabetes is a serious medical condition that can be treated. A recently completed study carried out by scientists in the United States conclusively showed that people with prediabetes can prevent the development of T2D by making changes in their diet and by increasing their level of physical activity. They may even be able to bring their blood glucose levels back to the normal range.

Lifestyle changes are of outmost importance. A balanced diet and an increase of the level of physical activity can help maintain a healthy weight, stay healthier for longer and reduce the risk of diabetes. The results of the Diabetes Prevention Program (DPP) proved that weight loss through moderate diet changes and physical activity can delay or prevent T2D (Haus, 2010). The DPP was a major multicenter clinical research study aimed at discovering whether modest weight loss through dietary changes and increased physical activity or treatment with the oral diabetes drug metformin (Glucophage) could prevent or delay the onset of T2D in study participants.

1.9.3.4 Lifestyle and T2DM

T2DM is due primarily to lifestyle factors and genetics (Ripsin et al., 2009). A number of lifestyle factors are known to be important to the development of T2DM. These are physical inactivity, sedentary lifestyle, cigarette smoking and generous consumption of alcohol (Hu et al., 2001). Obesity has been found to contribute to approximately 55% of cases of T2DM (CDC, MMWR, 2004). Environmental toxins may contribute to the recent increases in the rate of T2DM. A weak positive correlation has been found between the concentrations in the urine of bisphenol A, a constituent of some plastics, and the incidence of T2DM (Lang et al., 2008).

1.9.3.5 Genetics and T2DM

T2DM has been empirically shown to be a partially inheritable disease in which a genetic component plays a significant role in disease etiology. Genetic susceptibility plays a crucial role in the etiology and manifestation of T2D but the underlying pattern is complicated, since both impairment of β-cell function and an abnormal response to insulin are involved. Studying the genetics of T2DM has been difficult due to the complexity of this disease.

The following difficulties partially explain the reason of the slow progression of the genetic discoveries of complex T2DM (Hansen, 2005)-

i) Late onset of the disease;

ii) Increased morbidity and mortality resulting in incomplete pedigrees consisting of no more than two generations;

iii) Huge variations in phenotypes;

iv) Clinical and pathogenic heterogeneity;

v) Absence of a clear transmission pattern within the pedigrees.

However, through years of efforts, a few of the discovered genes were fully proven to influence the susceptibility to this complex disease. It is worth mentioning that this is not the same as a susceptibility gene. The term ‘susceptibility gene’ implies that a gene is not strong enough to act like a diabetogene on its own, but can only act as a pro-diabetic gene by interacting with other pro-diabetic genes, the metabolic environment of the body (e.g. glucotoxicity and lipotoxicity) and the life style (e.g. sedentary life, excess calories, smoking, stress and chronic inflammation) (Hansen, 2005).

1.10 DNA polymorphisms associated with type 2 diabetes

Many studies around the world have documented that genetic factors play a key role in the incidence of T2DM (Kahn et al., 1996; Taylor, 2006; McCarthy et al., 2002). Identification of these genetic elements relies primarily on genome wide linkage studies or candidate gene approach for the most common forms of T2DM. Previously, numerous candidate genes have been identified in the predisposition to T2DM; however, only a few have been replicated in different populations with various racial backgrounds (Hirschhorn et al., 2002). Previous linkage studies and candidate gene approaches have identified several genes associated with T2DM, such as *CAPN10, ENPP1, HNF4A, ACDC, PPARG, KCNJ11* and *SLC30A8* (Weedon et al., 2003; Hanis et al., 1996; Mohaddes et al., 2012). Whole genome association studies (WGAS) has also showed genetic variants in more than 15 genes/loci to be associated with T2D (Zeggini et al., 2007; Saxena et al., 2007; Scott et al., 2007).

1.11 Major polymorphisms associated with Type 2 diabetes

The genetic pre-disposition of T2DM can be known through the candidate gene approach in which variants of a particular candidate gene are analyzed based on its pathophysiology. Variants in genes encoding proteins that play a role in insulin control and glucose homeostasis pathways are excellent candidates for genetic pre-disposition of T2DM

(Dehwah et al., 2009). Recent insights into the molecular mechanisms of pancreatic development, insulin signaling, insulin secretion and adipogenesis and consequent physiologic changes in these and in other pathways, which were thought to contribute to diabetes, have lead to an explosion in the number of candidate genes for T2DM (Radha et al., 2003).

The genetic basis of T2DM is well illustrated in recent review articles (Dehwah et al., 2009; Radha et al., 2003; Das et al., 2006; Zeggini et al., 2007; Ridderstrale et al., 2009; Taylor et al., 2006). A list of candidate genes involved in the etiology of T2DM. Figure 1.3: represents a bar diagram showing number of studies with and without association between T2DM and different candidate genes.

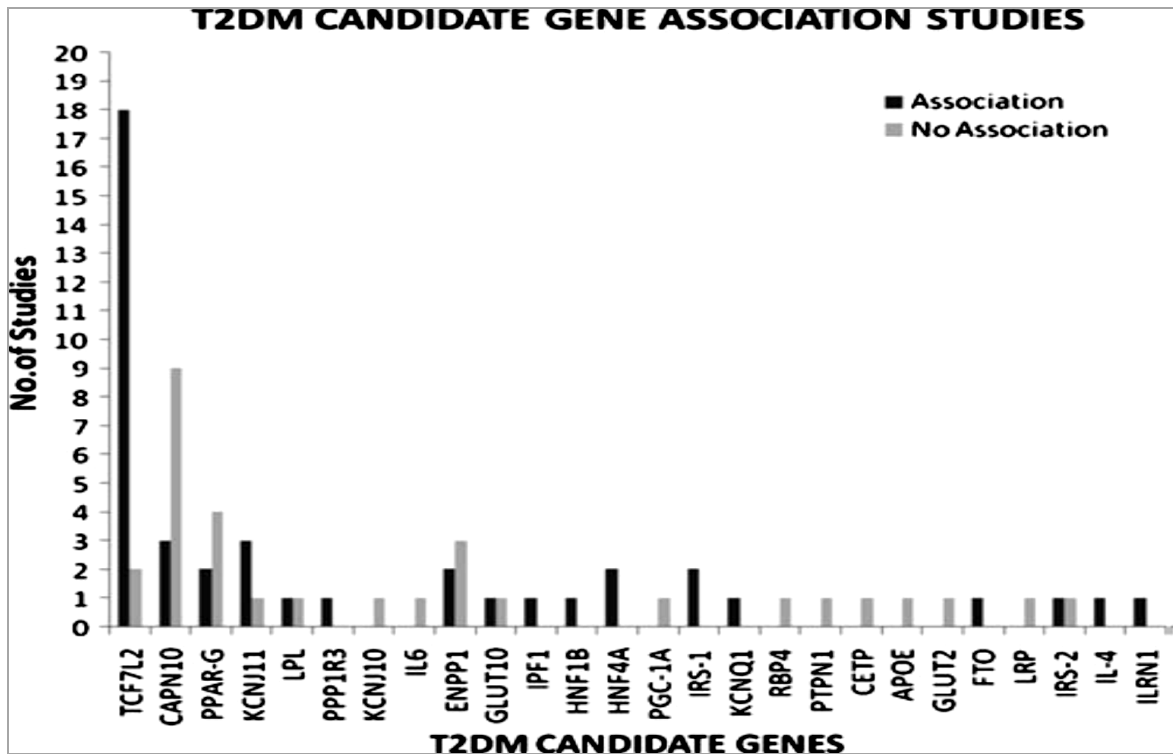


Figure 1.3: Represents a bar diagram showing number of studies with and without association between T2DM and different candidate genes (Kommoju, 2011).

Calpain10

The calpains are a family of Ca^{2+} -dependent, intracellular cysteine proteases. Calpains cause a limited proteolysis of a variety of substrates rather than extensive degradation, thus modulating substrate structure and activity. Calpains are involved in a wide range of physiological processes (e.g., cell motility, cell cycle, cellular differentiation, signal transduction pathways), and in pathological processes (e.g., neurodegenerative diseases, muscular dystrophies, cancer, gastropathies, diabetes mellitus). There are 15 calpain genes in the human genome coding for members of calpain family proteins.

CAPN10 gene is positioned on chromosome 2q37.3, which has a province that was previously described as a susceptibility gene for diabetes, termed NIDDM1 (non-insulin dependent diabetes mellitus 1). *CAPN10* gene consists of 15 exons and generating eight mRNA isoforms by alternative splicing (calpain-10a to h), though many of these isoforms are degraded by nonsense-mediated mRNA decay (NMD) (Horikawa et al., 2000; Green et al., 2003). Four main polymorphisms of *CAPN10* have been linked with diabetes: SNP-43 (rs3792267), SNP-44 (rs2975760), SNP-63 (rs5030952) and InDel-19 (rs3842570). These SNPs are restricted in intronic regions and do not influences the amino acid structure of the protein, but most likely modify the gene expression or alternative splicing mechanisms (Horikawa et al., 2000).

The identification of the calpain-10 gene (*CAPN10*) as a putative diabetes gene suggests a new biochemical pathway involved in the regulation of blood glucose levels. This gene was first found to be associated with risk of type 2 diabetes in studies carried out in Mexican Americans (Horikawa et al., 2000). Variation in the calpain-10 gene (*CAPN10* [MIM 605286]) was recently linked and associated with type 2 diabetes mellitus (T2DM) susceptibility (Horikawa et al., 2000).

ENPP1

Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (*ENPP1*; also known as plasma cell membrane glycoprotein 1 or PC-1) belongs to a family of *ENPP* enzymes that regulate pyrophosphate and nucleotide levels. PC-1 reduces both insulin receptor function

and subsequent downstream signalling, and has a wide range of tissue expression that includes skeletal muscle and the liver (Harahap et al., 1988; Kumakura et al., 1998). Furthermore, protein levels of PC-1 are increased in insulin-resistant subjects and correlate with whole-body insulin resistance (Frittitta et al., 1996). Overexpression of human PC-1 in the liver of mice induces insulin resistance and glucose intolerance (Dong et al.,2005), suggesting that this protein influences insulin sensitivity and thereby may affect the risk of developing type 2 diabetes. The involvement of PC-1 in insulin-resistant states is further supported by recent studies revealing evidence for linkage between the chromosomal region 6q22–q23 of the gene encoding PC-1 (*ENPP1*) and insulin resistance (Duggirala et al., 2001), obesity (Meyre et al., 2004; Atwood et al., 2002) and type 2 diabetes (Ehm et al., 2000; Ghosh et al., 2000; Demenais et al., 2003; Xiang et al.,2004). Furthermore, the minor Q allele of a K121Q variant (rs1044498) of *ENPP1* has been shown to influence PC-1 protein function by inhibiting insulin receptor function and insulin signalling more effectively than the major K allele (Pizzuti et al., 1999; Costanzo et al., 2001). Follow-up genetic association studies found that the minor Q allele of K121Q increases the risk of type 2 diabetes in a Dominican population (Hamaguchi et al., 2004), in South Asians living in the U.S. and in India, and in U.S. white samples (Abate et al., 2005); it has also been associated with earlier onset of type 2 diabetes and myocardial infarction in other populations of European ancestry (Bacci et al., 2005).

KCNJ11

Adenosine triphosphate (ATP)–sensitive potassium ion channel (KATP) plays a key role in insulin secretion by glucose-stimulated pancreatic β-cells (Ashcroft et al., 1984). Potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*), which encodes the subunit protein of KATP (Kir 6.2), is highly expressed in the pancreas. Variations in the *KCNJ11* E23K gene could contribute to the decreased sensitivity of the ion channel to ATP, which makes the channel consume more ATP until it is closed. Thus, insulin release is impaired and the T2D risk increases.

KCNJ11 gene, located at 11p15.1, spans 2 kb and contains 1 exon that encodes 390 amino acids (Inagaki et al., 1995). *KCNJ11* E23K gene polymorphism is located in the 1st exon

and formed by missense mutation of the 23rd codon. The substitution mutation of adenine (A base) for guanine (G base) in the 23rd codon replaces glutamine (E) with lysine (K) in the corresponding amino acid sequence.

Although *KCNJ11* E23K gene polymorphism has been indicated in T2D susceptibility, research results remain debatable worldwide. In 2003, Gloyn et al. (Gloyn et al., 2003) evaluated the role of the *KCNJ11* E23K variant by studying 854 T2D and 1,182 control subjects from the UK and found that the 23 K allele is associated with diabetes. Moreover, the meta-analysis of all the case–control data showed that the E23K allele is associated with T2D. In 2007, Koo et al. (Koo et al., 2007) also found that the polymorphic loci of *KCNJ11* E23K are strongly associated with T2D in the Korean population. In contrast, Nielsen et al. (Nielsen et al., 2003) found that the association of the *KCNJ11* E23K polymorphism with T2D is not significant in Denmark. In 2006, Yokoi et al. (Yokoi et al., 2006) reported that the E23K variant of *KCNJ11* showed no association with diabetes among the Japanese.

Studies on the association between *KCNJ11* E23K gene polymorphism and T2D were performed extensively in China, but the results remain controversial. In 2009, Wang et al. (Wang et al., 2009) reported that the *KCNJ11* E23K gene polymorphism is associated with T2D. However, in 2011, Wang et al. (GUI-FENG et al., 2011) reported that no association was found between the *KCNJ11* E23K gene polymorphism and T2D.

PPARG

PPARG activates the expression of genes involved in glucose and lipid metabolism, which converts nutritional signals into metabolic consequences (Ryan et al., 2011). *PPARG* is arguably the first gene identified for the complex late onset form of T2D via a candidate-gene approach. The *PPARG* Pro12Ala (rs1801282) has been the most vastly investigated single-nucleotide polymorphism (SNP), which was believed to alter transcriptional activity as a result of its location in the functional binding domain that has been associated with risk of T2D and its intermediate traits (Gaulton et al., 2008; Gouda et al, 2010; Herder et al., 2008 ; Lin et al, 2010; Lyssenko et al., 2008; Meigs et al., 2007; Sanghera et al., 2008; Wen et al., 2010). Since its first report in 1997 assessing the effect of the Pro12Ala

(rs1801282) variant on T2D risk, majority of the approximately 60 association studies have confirmed the relation of this particular variant to T2D risk, mainly in Caucasians. However, there appears to be some statistically significant heterogeneity across these studies [I^2=37%, 95% confidence interval (CI) 9%–54%; P=.0028] (Gouda et al, 2010). In particular, *PPARG* was not consistently confirmed to be significant in non-Caucasian populations, e.g. a genome-wide association study (GWAS) conducted in African Americans (Palmer et al., 2012), a multi stage GWAS meta-analysis in East Asians (Cho et al., 2012), or a multi stage GWAS meta-analysis in South Asians (Kooner et al., 2011).

WFS1

Wolfram syndrome (*WFS1*) is a rare, progressive, neurological disorder with an autosomal-recessive mode of inheritance, which frequently manifests in childhood (Barrett et al. 1995). Diabetes insipidus and (non-autoimmune) diabetes mellitus with optic atrophy and deafness are features of the syndrome, giving rise to its alternative name: DIDMOAD.

Positional cloning studies in families with Wolfram syndrome identified linkage peaks on the short arm of chromosome 4 (4p16.1) (Polymeropoulos et al. 1994) and mutations in the gene encoding wolframin (*WFS1*), which maps to that region, have since been shown to cause the syndrome (Inoue et al., 1998).

In a recent report, four common single nucleotide polymorphisms (SNPs) (rs10010131, rs6446482, rs752854 and rs734312 [H611R]) at the *WFS1* locus were shown to be convincingly associated with type 2 diabetes in six UK studies and one study of an Ashkenazi Jewish population (Sandhu et al., 2007). (Gaulton et al., 2008; Gouda et al., 2010; Herder et al., 2008 ; Lin et al., 2010; Lyssenko et al., 2008; Meigs et al., 2007; Wen et al., 2010)

HNF4

Hepatocyte nuclear factor-4α (*HNF4α*) gene variants have been shown to co segregate in an autosomal-dominant manner in families with an atypical form of type 2 diabetes known as maturity-onset diabetes of the young (MODY)-1. MODY is a clinically and genetically

heterogeneous form of non ketotic diabetes that presents before age 25 years, usually in non obese, asymptomatic, hyperglycemic individuals (Winter, 2003; Stride et al., 2002; Fajans et al., 2001). *HNF4α*'s role in MODY stems from its function as a-cell transcription factor that influences glucose-induced insulin secretion (Byrne et al., 1995). In contrast to MODY, type 2 diabetes usually occurs between ages 40 and 60 years, with the exception of obesity-related pediatric type 2 diabetes, regardless of family history (Aye et al., 2003). Both MODY and type 2 diabetic patients have reduced insulin sensitivity as a result of pancreatic islet β-cell dysfunction. In addition, *HNF4α* has been shown to influence lipid transport and metabolism (Bartoov-Shifman et al., 2002; Wang et al., 2000). *HNF4α* is differentially expressed in mammalian liver, kidney, small intestine, colon, stomach, and pancreas from as many as nine different transcripts (Nakhei et al., 1998; Boj et al., 2001). An alternative promoter, P2, lies 45.6 kb upstream of the proximal P1 promoter (Boj et al., 2001; Thomas et al., 2001; Hansen et al., 2002). P2-driven transcripts have been described as the predominant splice variant in pancreatic β-cells (Boj et al., 2001; Thomas et al., 2001; Hansen et al., 2002; Eeckhoute et al., 2003). Although HNF4α intragenic and/or proximal P1 promoter single nucleotide polymorphisms (SNPs) have been described in previous type 2 diabetes studies (Moller et al., 1997; Hani et al., 1998; Price et al., 2000; Malecki et al., 1998; Sakurai et al., 2000), a thorough examination of the P2 region has not been reported; thus, association mapping was designed to examine the P2 region in this study.

1.12 Transcription factor 7-like 2 (*TCF7L2*)

Transcription factor 7-like 2 (*TCF7L2*, formerly called TCF4) is a member of the T-cell-specific high-mobility group (HMG) box-containing family of transcription factors that plays a key role in the WNT signaling pathway (Jin, 2008), involved in lipid metabolism and glucose homeostasis. Illustrated in Figure 1.4, the gene spans 215.9 kb and comprises 17 exons. The gene possesses two major domains: a catenin-binding domain (exon 1) and a central DNA-binding HMG domain (exons 10 and 11) (Poy et al., 2001). At least five exons can be alternatively spliced (Duval A. et al, 2000) and most human tissues express detectable levels of this transcription factor (Cauchi et al., 2006). Although the T2D-associated SNPs are located in non-coding regions it is not clear if these SNPs, or a variant

in strong linkage disequilibrium (LD) with these, play a role in alternative splicing, gene expression, or protein structure.

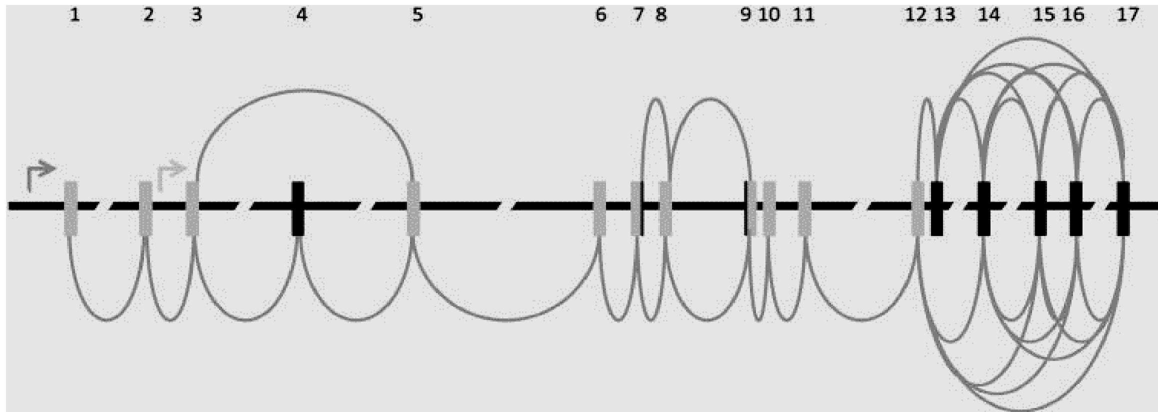


Figure 1.4: Structure of the *TCF7L2* gene. Arrows indicate transcription start sites. Grey bars indicate exons. Black bars indicate alternatively spliced exons. Round lines between exons indicate alternative splicing products (Pang et al., 2013).

The WNT signaling pathway has been well known to be associated with the developmental pathways such as embryogenesis including adipogenesis and pancreatic islet development, and tumorigenesis (Christodoulides et al., 2009; Heller et al., 2002; Welters et al., 2008; Angus-Hill et al., 2011). Activation of this pathway leads to accumulation of β-catenin in the nucleus, which interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to regulate the transcription of WNT target genes, many of which are associated with the cell proliferation and cell fate decision (Jin, 2008; Liu et al., 2011).

Besides the developmental role, several lines of evidence suggest the role of WNT signaling pathway in the etiology of metabolic disorders (Kanazawa et al., 2004; Christodoulides et al., 2006; Mani et al., 2007; Salpea et al., 2009). Particularly, single nucleotide polymorphisms (SNPs) in human *TCF7L2* gene are known to be strongly associated with an increased risk of type 2 diabetes through the extensive genome-wide association studies in multiple ethnic populations (Grant et al., 2006; Florez et al., 2006; Scott et al., 2007). *TCF7L2* variants have been associated with an impaired β-cell function including the impaired insulin secretion and processing, an increased insulin resistance, and hyperglycemia (Bonetti et al., 2011; Strawbridge et al., 2011; Lyssenko et al., 2007).

1.12.1 Location of *TCF7L2* gene

Cytogenetic Location: 10q25.3

Molecular Location on chromosome 10: base pairs 112,950,219 to 113,167,678

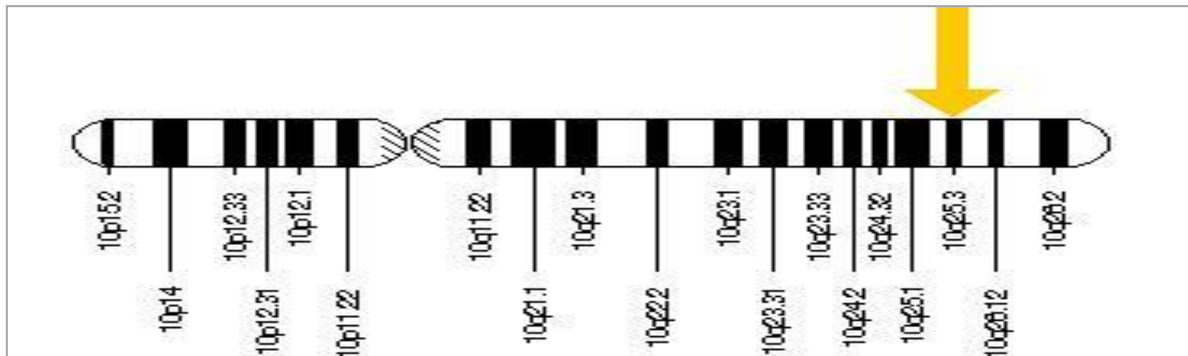


Figure 1.5: Location of *TCF7L2* gene on the long (q) arm of chromosome 10 at position 25.3.

1.12.2 The association of genetic variants in *TCF7L2* withT2DM

Among the large number of candidate genes, *TCF7L2* emerges as one of the most promising genes in T2DM susceptibility. (Figure 1.6) presents the overall structure of the human *TCF7L2* gene, the position of the microsatellite DG10S478, and the positions of the five SNPs that were initially investigated by Grant et al. , as well as two SNPs identified in studies from Taiwan (rs290487) and Hong Kong (rs11196218) (Ng et al., 2007 and Chang et al., 2007).

The strongest common variant associated with T2DM identified to date is rs7903146 in the *TCF7L2* gene, which has a per allele odds ratio of~1.35 for risk of the disease (Scott et al., 2007; Sladek et al., 2007; Zeggini et al., 2007). The first signal detected in the gene was a microsatellite, identified using the linkage study. In 2006, Grant et al. reported that a microsatellite (DG10S478) in *TCF7L2* was associated with an increased risk of T2DM (Grant et al., 2006). The DG10S478 is located in intron 3of *TCF7L2*. Two SNPs investigated within intron 3 and 4 of *TCF7L2* (rs7903146 and rs12255372) were in strong linkage disequilibrium with DG10S478 and showed similar robust associations with T2DM (Grant et al., 2006). Subsequent studies in many other ethnic groups have confirmed thatrs7903146 and rs12255372 are the two SNPs most strongly associated with

T2DM (Saxena et al., 2006; Helgason et al., 2007). Several other SNPs in the 3′-end of *TCF7L2* have also been associated with T2DM in an East Asian population (Cho et al., 2012). Since 2006, numerous studies have tried to functionally link the risk T-allele ofrs7903146 with T2DM by investigating the *TCF7L2* expression level, alternative splicing (AS) and the physiological function of *TCF7L2* in pancreas and liver. Most of the 65 common variant signals identified by GWAS are associated with impaired pancreatic islet function, indicating that pancreas could be the primary target tissue of the *TCF7L2*-associated risk of developing T2DM (Voight et al., 2010; Cho et al., 2012).

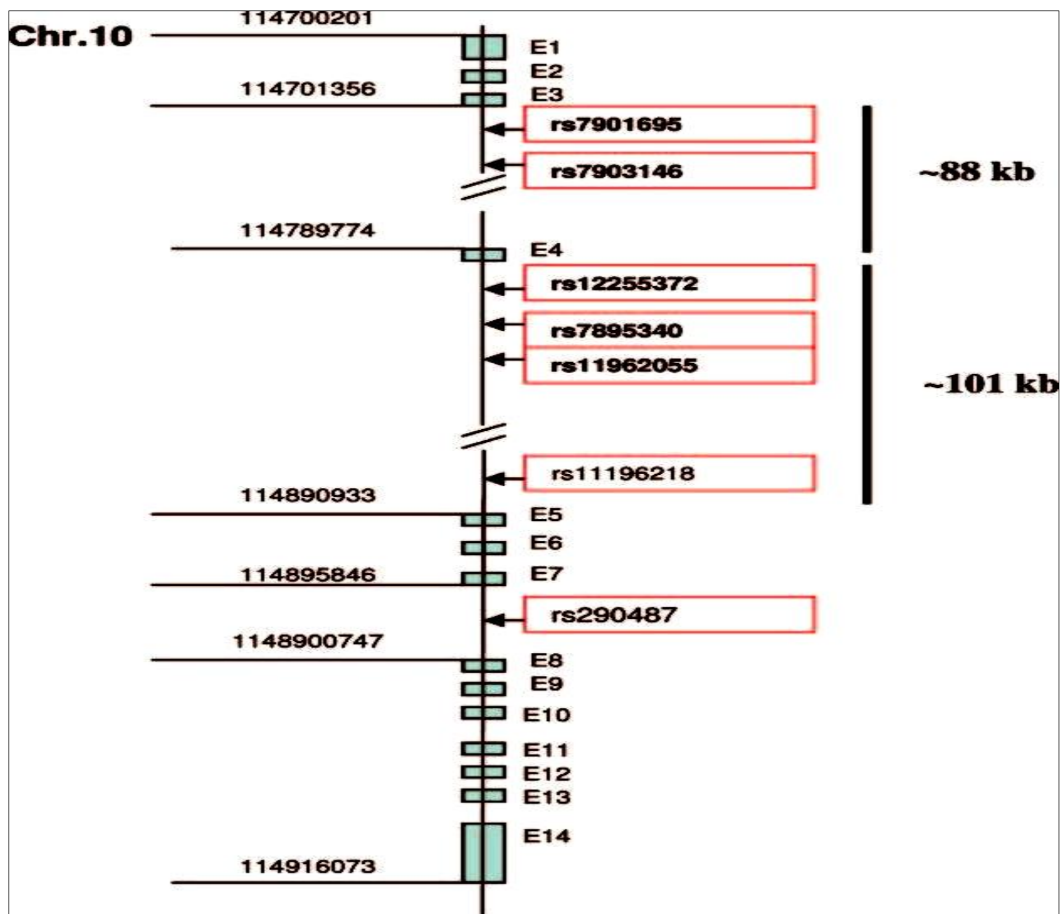


Figure 1.6: The Diagram Shows the Positions of Seven SNPs and the Microsatellite DG10S478 in the Human *TCF7L2* Gene Among the seven SNPs, five (in bold) were initially studied by Grant et al. (Grant et al, 2006). The SNP rs290487 is the one that was identified by Chang et al. (Chang et al, 2007) in a Chinese population study. The SNP rs11196218 is the one that was identified by Ng et al. (Ng MC.et al, 2007) in a Hong Kong Chinese study. Chr.10, Chromosome 10; E1–14, exons 1–14. (Jin & Liu, 2008).

1.12.3 *TCF7L2*-a critical component of WNT signaling and action

TCF7L2 is a nuclear receptor for CTNNB1 (previously known as β-catenin), which in turn mediates the canonical WNT signalling pathway. The WNT signalling pathway is critical for normal embryogenesis, cell proliferation and motility, as well as cell fate determination. Mutations in different molecules involved in WNT signalling have been identified in several cancers, and other disruptive mutations are associated with decreased bone mass (Polakis et al., 2000; Boyden et al., 2002). WNT signalling is also critical for the normal self-renewal of stem cells, as well as in regulating myogenesis and adipogenesis (Etheridge et al., 2004; Ross et al., 2000). In addition, tightly regulated WNT signalling is required for the normal development of the pancreas and islets during embryonic growth (Papadopoulou et al., 2005).

(Figure 1.7) Schematically summarises the canonical (β-catenin-related) WNT signalling pathway. WNTs are ligands secreted by different cells; 19 WNTs have been identified, illustrating the complexity of this signalling pathway, as well as its potential for specificity. WNTs bind to FZD (previously known as Frizzled) and lipoprotein- related protein (LRP) receptors, which, in turn, prevent glycogen synthase kinase 3β (GSK3B) from phosphorylating β-catenin, thereby preventing its degradation and increasing the cellular levels of this activator. In the absence of secreted WNTs, β-catenin is rapidly phosphorylated by GSK3B and degraded. In the presence of WNTs, the increased β-catenin binds to the nuclear receptor *TCF7L2*, which then leads to its activation and subsequent induction of many different genes and proteins involved in cell proliferation and differentiation. It is obvious, considering the profound effects of WNT signalling, that this pathway needs to be tightly regulated.

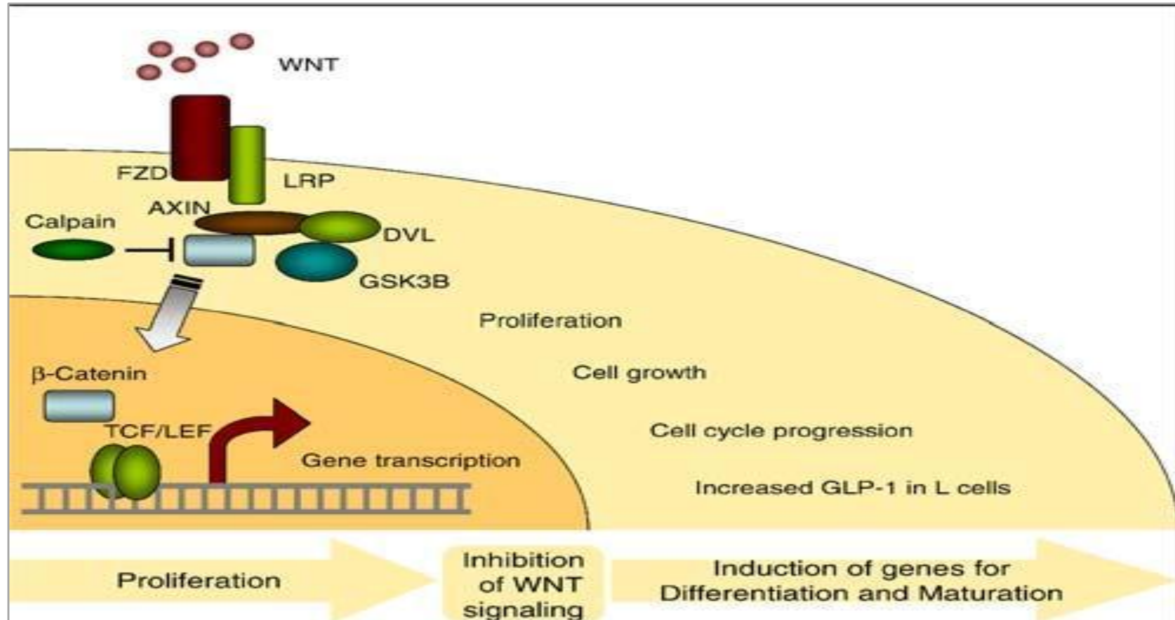


Figure 1.7: Schematic representation of the canonical WNT signaling pathway, which is fundamental for growth and development. (Smith, 2007).

1.12.4 How is WNT signaling related to type 2 diabetes

There are several known mechanisms for the involvement of WNT signaling in both insulin secretion and action, as well as in cell differentiation and maturation.

WNT signaling through the *TCF7L2* nuclear receptor has been shown to be critical for glucagon-like peptide-1 (GLP-1) secretion by the intestinal endocrine L-cells (Yi et al., 2005). Thus, an alteration in this pathway could lead to a reduced secretion of GLP-1 which, in turn, could have consequences for both the insulin secretion following a meal and the generation of new beta cells from the ductal precursor cells. The consensus finding that the *TCF7L2* risk variants are associated with a reduced insulin secretion supports such a possibility.

However, the recent finding (Cauchi et al., 2006) that the *TCF7L2* gene is also expressed in human pancreas, in apparent contrast to murine models (Yi et al., 2005), suggests direct effects on normal beta cell insulin secretion or, more likely, beta cell growth and differentiation from the precursor cells.

1.12.5 *TCF7L2*, its polymorphisms and type 2 diabetes

More than a decade ago, an investigation by Duggirala and colleagues (Duggirala et al., 1999) showed that a region on chromosome 10q was associated with T2D in Mexican Americans. A later study also found a linkage between T2D and chromosome 10q in an Icelandic population (Reynisdottir et al., 2003). These studies found significant evidence that a susceptibility locus near the marker D10S587 on chromosome 10q was strongly linked with T2D. This was then followed by genome-wide association (GWA) studies. At least 19 genes have been identified to date that are associated with T2D, from several different populations, from GWA studies (Cauchi et al., 2007; Grant et al., 2006; Lyssenko et al., 2007; Scott et al., 2006; Sladek et al., 2007). Many of these genes are expressed in pancreatic β-cells, and are involved in the WNT signaling pathway. It has been reported that among the four members of the *TCF/LEF* family, *TCF7L2* is the major partner of β-catenin in intestinal epithelia. Followed the initial investigation by Grant et al. in 2006 (Grant et al., 2006), extensive genome-wide association (GWA) studies have confirmed a relationship between certain polymorphisms in the *TCF7L2* gene and the risk of developing T2D. Grant and colleagues (Grant et al., 2006) followed up a T2D linkage signal on chromosome 10q in Icelandic populations and found a strong association between T2D status and *TCF7L2* polymorphisms within intronic regions. This group has genotyped 228 microsatellite markers in individuals with T2D and healthy controls across a 10.5-Mb interval on chromosome 10q. Microsatellite, DG10S478, located within intron 3 of the *TCF7L2* gene, was found to be associated with T2D. This observation was replicated by the same group in populations from the USA and Denmark (Grant et al., 2006). It was found that two of five single nucleotide polymorphisms (SNP), namely rs12255372 and rs7903146, were in strong linkage disequilibrium with the marker DG10S478 and also indicated similar robust associations with T2D (Grant et al., 2006). The observation that *TCF7L2* SNPs are linked with T2D has been replicated and expanded upon by later studies (Sladek et al., 2007; Florez et al., 2006). Florez and colleagues (Florez et al, 2006) investigated whether the two SNPs, rs12255372 and rs7903146, were linked with diabetes progression individuals with impaired glucose tolerance in a diabetic prevention program. They observed that these common polymorphisms in *TCF7L2* are

associated with an increased risk of diabetes among subjects with impaired glucose tolerance (Florez et al., 2006).

In addition to SNPs rs12255372 and rs7903146, there are also several other SNPs on chromosome 10q that have been identified as associated with an increased risk for T2D (Chang et al., 2007; Ng et al., 2007). In Asian populations, the frequencies of these two SNPs are relatively low, although a linkage between these two SNPs and T2D has been reported in two large Japanese populations

The following *TCF7L2* SNPs, already known to be risk loci of T2DM, were genotyped: rs7901695, rs7903146, rs11196205, rs12255372. The risk alleles of 3 (rs7901695, rs7903146, rs11196205 SNPs were associated with higher fasting plasma glucose ($p<0.01$, $p<0.03$ and $p<0.01$ respectively). The risk alleles of the first two SNPs (rs7901695, rs7903146) were associated to a decrease in the proportional control of β-cell function, $p<0.02$ and $p<0.03$ respectively (Hayashi et al., 2007; Horikoshi et al., 2007). Several variants of *TCF7L2* which are associated with T2DM but rs7901695 is a most significant genetic marker which is associated with T2DM in South Asian population. Chang and colleagues (Chang et al., 2007) genotyped SNPs across the *TCF7L2* gene in a Han Chinese population in Taiwan and identified a novel SNP, rs290487, linked to T2D. More recently, Ng et al. (Ng et al., 2007) identified a relationship between another novel SNP, rs11196218, and an increased risk in T2D in a Hong Kong Chinese study. However, whether these SNP alleles associated with T2D in other populations requires further investigation. Taken together, these observations suggest that certain specific polymorphisms in *TCF7L2* are in strong associations with an increase risk of developing T2D.

1.13 Mutation and polymorphism

Mutation is often defined as any change in a DNA sequence away from normal. This implies there is a normal allele that is prevalent in the population and that the mutation changes this to a rare and abnormal variant. In contrast, a polymorphism is a DNA sequence variation that is common in the population. In this case no single allele is regarded as the standard sequence. Instead there are two or more equally acceptable alternatives. The arbitrary cut-off point between a mutation and a polymorphism is 1 per

cent. That is, to be classed as a polymorphism, the least common allele must have a frequency of 1% or more in the population. If the frequency is lower than this, the allele is regarded as a mutation.

1.13.1 Factors relating to polymorphism

Polymorphism arises by mutation. Several factors may maintain polymorphism in a population.

- Polymorphism can be maintained by a balance between variations created by new mutation and natural selection.
- Genetic variation may be caused by frequency-dependent selection.
- Multiple niche polymorphisms exist when different genotypes should have different fitness in different niches.
- Heterozygous advantage may maintain alleles which would otherwise be selected against.
- If selection is operating, migration can introduce polymorphism into a population.

These are all sources of polymorphism which make use of the mechanisms of natural selection. Genetic drift is also a possible source of genetic variation.

1.13.2 Role of Genetics polymorphism in the Development of DM

People with a family history of T1DM and T2DM are six and three times more likely, respectively, to develop these diseases than are unrelated individuals. Multiple genes are involved in DM. Those that have garnered the most attention are the ATP-binding cassette transporter subfamily C member 8 (*ABCC8*) gene; the *KCNJ11* gene; and the peroxisome proliferator-activated receptor-gamma (*PPARG*) gene. Most of these genes are involved in insulin action/ glucose metabolism, pancreatic beta cell function or other metabolic conditions (e.g., energy intake/expenditure, lipid metabolism). Mutations in genes such as *ABCC8* and *KCNJ11* can disrupt the potentiating activity of the KATP channel and have

thus been associated with permanent neonatal DM. The *PPARG* gene is implicated in adipogenesis and the development of insulin resistance. Deleterious mutations in this gene impair insulin resistance and can cause lack of response to insulin.

From recent genome-wide association studies, more than 60, 500, and 65 loci have been identified for susceptibility to T1DM, T2DM, and GDM, respectively. SNPs are the most common type of genetic variation distributed within or outside a gene region in the human genome. The frequency of SNPs is less than 1% in the genome, and approximately 54% of these variants are not deleterious. SNPs can modify the risk of occurrence of a disease, either alone or in linkage disequilibrium in one gene or in neighborhood genes. For instance, in several studies, the common Pro12Ala polymorphism in the *PPARG* gene, the Glu23Lys polymorphism in the *KCNJ11* gene, or the Ser1369Ala polymorphism in the ABCC8 gene was confirmed to be associated with DM.

1.14 Methods for mutation detection

The varieties of mutations found in the human genome can range from the alteration of a single base to major rearrangements affecting large parts of or whole chromosomes. The existence of different types of mutation therefore means that diverse approaches to mutation detection are required. Mutation detection procedures can thus be tailored to the particular gene of interest if such a predisposition exists or designed to detect virtually all types of mutation for a more universal approach. A reliable, cost effective and rapid scanning analysis for the *TCF7L2* gene is required, in order to obtain trustworthy results. Denaturing gradient gel electrophoresis (DGGE) analysis, Restriction Fragment Length Polymorphism (RFLP), denaturing high performance liquid chromatography (DHPLC), single strand conformational polymorphism analysis (SSCP) and Sanger Sequencing are scanning/screening methods that have been applied thus far, but although highly reliable, they are laborious and time consuming. On the other hand, high-resolution melting (HRM) analysis, which represents the next generation of mutation scanning technology, can easily be adapted in a diagnostic laboratory. The method relies on the ability to distinguish alterations in composition, length, GC content, or strand complementarity of DNA sequences.

1.15 High Resolution Melt (HRM) analysis

Real-time polymerase chain reaction (RT-PCR) is an ideal platform for high-throughput genotyping (Price et al., 2007). Recent developments involve the coupling of RT-PCR with HRM, using a single instrument (Stephens et al., 2008). High resolution melting (HRM) analysis is a refinement of melting curve analysis that was developed in 2002 by collaboration between industries (Idaho Technology. UT, USA) and academics (University of Utah, UT, USA). It is able to identify smaller differences in PCR amplicons down to the single base level and is therefore ideal for single nucleotide polymorphism genotyping, species identification, sequence matching and mutation scanning without the need for any further separation and additional processing after PCR (Reed et al., 2007; Erali et al., 2008).

In HRM does not require the use of labelled probes using instead fluorescent DNA intercalating dye (eg SybrGreen) is monitored during strand dissociation events during the melt phase. The dyes are added to the PCR reaction mixture. After PCR, in order to generate a melting curve (MC), the PCR products are heated with different ranges of temperature starting from lower temperature and gradually increase (From 40°C to 95°C). The melting temperature of the amplicon is a characteristic of sequence, length and GC content, and is the temperature at which 50% of DNA is in a double strand (Reed et al., 2007; Erali et al., 2008; Herrmann et al., 2006). The PCR amplicon is subjected to small temperature increments. At lower temperature, any double stranded DNA in the sample will give a high fluorescence but when temperature increases, the double stranded DNA is separated into a single stranded DNA releasing the dye and a drop in fluorescence can be observed (Erali et al., 2008). While melt curves are principally used to establish the melting temperature (T_m) of an amplified DNA fragment, the shape of the melt curve is representative of the DNA sequence undergoing melting, allowing amplicons to be distinguished based on their melt curve shape, even if they share the same Tm value (Stephens et al., 2008). Shape differences in the melting curves can be displayed with normalization and comparison software (Price et al., 2007), allowing the degree of sequence relatedness to be observed from which the sequence relatedness can be inferred. The high resolving power of HRM means that even a single polymorphism can influence

the melt curve, facilitating the detection of SNPs (Price et al., 2007; Reed et al., 2007). In comparison to other counterparts, the advantages associated with HRM make it a superior method for genotyping studies. This method is quicker, cheaper and is performed on a generic instrument. No separation or processing of samples is required and a closed-tube method decreases the risk of contamination (Reed et al., 2007). HRM has been used traditionally in gene mutation analysis (Dufresne et al., 2006; Graham et al., 2005).

Melting Curve Analysis

The extent of melting was measured by the change in fluorescence of the DNA sample. At low temperatures, the DNA was double-stranded and the dye strongly fluoresced. As the temperature increased, fluorescence decreased 1,000-fold as the dye was released when the two strands denatured. This decrease in fluorescence started slowly; but when the double-stranded DNA melts into its single-stranded, fully denatured form, a sharp decrease in fluorescence was detected. The rate of fluorescence decrease was generally greatest near the melting temperature (T_m) of the PCR product.

In control samples, Melt curves that are similar in shape and all are compact in position but that are distinguishable from each other by difference in T_m of the amplicon. Typically such profiles are generated by homozygous variant samples that are being compared to a wild type sample. In such situations, the Tm difference between samples is due to sequence variation from the wild type. Melt curves displaying a distinct curve shape from homozygote melt curves are usually due to the presence of base pairing mismatches (hetero duplexes) present in the PCR product mix.

Detection of wild type, heterozygote or homozygote

Organisms contain two (or more) copies of each gene, known as the two alleles. So, if a sample is taken from a patient and amplified using PCR both copies of the region of DNA (alleles) of interest are amplified. So if we are looking for mutation there are now three possibilities:

i. Neither allele contains a mutation

ii. One or other allele contains a mutation

iii. Both alleles contain a mutation.

These three scenarios are known as "Wild type", "Heterozygote" or "Homozygote" respectively. Each gives a melt curve that is slightly different. With a high quality HRM assay it is possible to distinguish between all three of these scenarios.

Usually all control samples are found in a compact position (Figure 1.8). Homozygous allelic variants may be characterized by a temperature shift on the resulting melt curve produced by HRM analysis. In comparison, heterozygotes are characterized by changes in melt curve shape. This is due to base-pair mismatching generated as a result of destabilized heteroduplex annealing between wild-type and variant strands. These differences can be easily seen on the resulting melt curve and the melt profile differences between the different genotypes can be amplified visually via generating a difference curve.

To detect mutation in different amplicons, normalized melting curve are presented in the (Figure 1.9). Melting curve analysis was performed using Eco Illumina software.

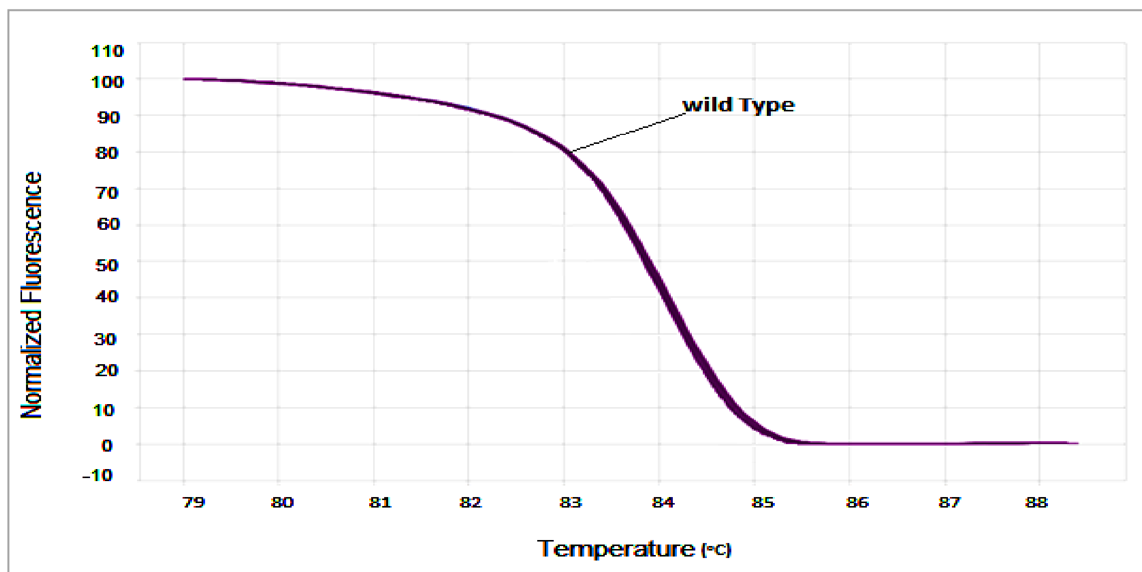


Figure 1.8: Normalized melting curve in control sample (Taylor et al. 2010)

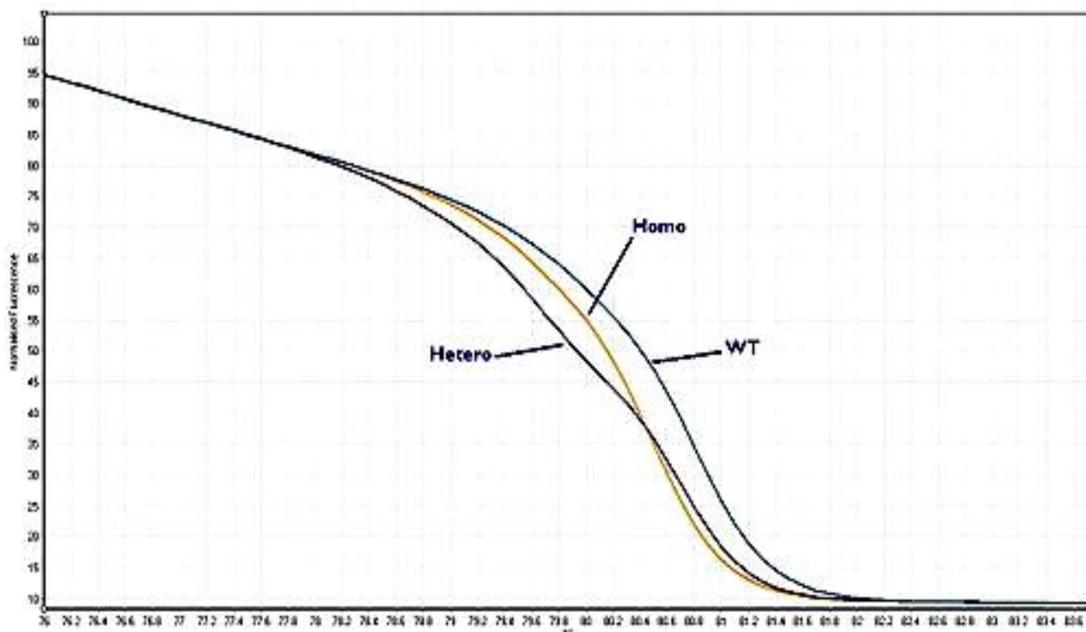


Figure 1.9: Normalized melting curve showing polymorphisms (Taylor et al. 2010).

Here a melting curve for non diabetic patients with no diabetic in family was considered as wild type. Curve from patients who have no mutation in any allele are almost aligned with wild type curve. Heterozygous mutated patient melting curve show distinct temperature shifting from wild type due to nucleotide variation in one of two alleles. Moreover, patients having homozygous mutation display a similar pattern of melting curve with wild type due to nucleotide variation in two alleles. The difference in melting temperature was identified with the difference between fluorescence of each curve and wild type curve.

Examples of different HRM curves

Here are some examples of different HRM curves which are found from different research work. Analyzing these curves we can identify the different mutation types (Figure 1.10).

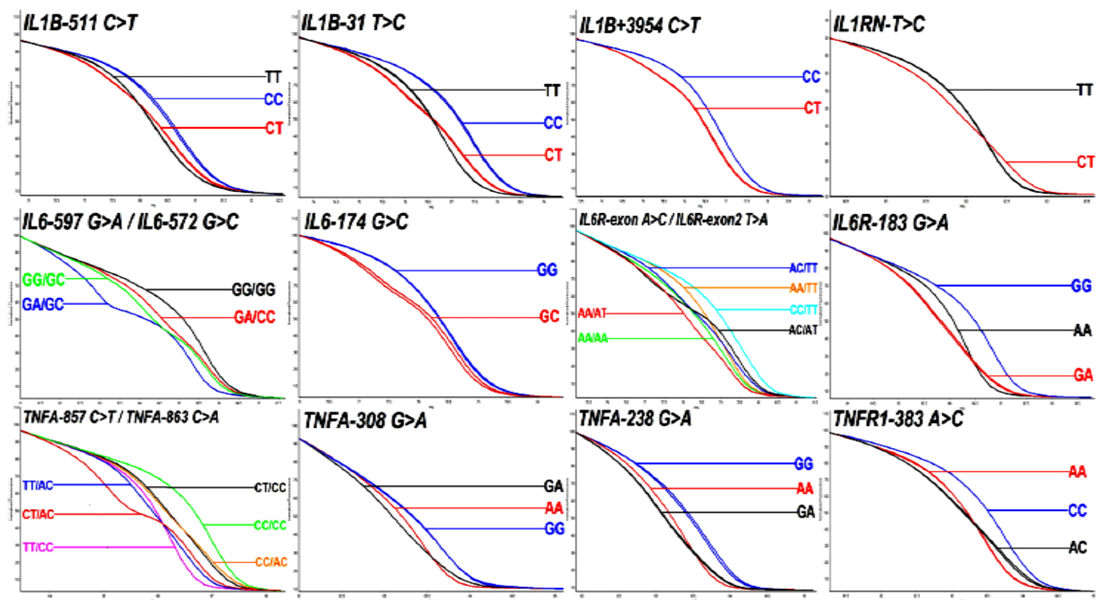


Figure 1.10: Examples of different polymorphisms showing the possibilities of different melting curves (You et al., 2013).

1.16 Diabetes in South Asia

South Asia constitutes one-fifth of the world's population and includes nine countries, all of which are undergoing lifestyle transitions that make their populations more vulnerable to develop T2DM. In the last few decades, the diabetes prevalence in South Asia has also risen considerably. South Asian populations also have a high prevalence of prediabetes and a more rapid progression to diabetes (Misra et al., 2014). This is highlighted by a recent study that reported a diabetes incidence of 22.2 per 1,000 person-years and that 59% of those with prediabetes converted to diabetes after a follow-up of 9.1 years (Anjana et al., 2015).

A 2008 report from Southern India showed a marked increase in diabetes prevalence in both urban and rural areas compared with earlier studies (Ramachandran et al., 2008). Studies from other parts of India have also shown increases in diabetes prevalence (Misra et al., 2014; Anjana et al., 2011).

India is the largest country in the region and has more than 65.1 million people with diabetes, occupying the second position next to China in the IDF global list of top 10

countries for people with diabetes. Pakistan and Bangladesh are in 12th and 13th positions, respectively (Federetion, 2013). The IDF estimates that the number of people with diabetes in South Asia will increase to 120.9 million, 10.2% of the adult population, by 2030. The highest increase in diabetes prevalence is noted in Mauritius (Magliano et al., 2011), an Indian Ocean island with a predominantly Asian Indian population. A survey conducted in urban and rural Maldives showed that the prevalence of diabetes was 10.6% (Aboobakur et al., 2010). However results from the STEPS survey conducted in Male, the Maldives capital city, showed the prevalence of diabetes was 4.5% in all adults. Again, the reason for the difference seen in the STEPS study is unclear.

Several distinctive features are noted among the South Asians, such as early occurrence of diabetes at lower BMI levels (Ramachandran et al., 2010), higher rates of insulin resistance, abdominal obesity, and familial aggregation of diabetes than in many other ethnic groups. In recent decades, increasing diabetes prevalence has been reported in rural areas in middle-income groups and among underprivileged people (Misra et al., 2014; Jayawardena et al., 2012). Occurrence of T2DM at a young age is commonly observed among South Asians, with the picture further complicated by the presence of maturity-onset diabetes of the young or latent autoimmune diabetes of adulthood (Misra et al., 2014).

Urban-Rural Difference

In developing countries in Asia and WPR, the urban-rural difference in diabetes prevalence is narrowing due to the increasing reach of Western lifestyles and associated behavioral changes into rural settings. In India, Nepal, Sri Lanka, the Solomon Islands, Samoa, and Thailand, more than 50% of the national population with diabetes resides in rural areas (Federation, 2013). Recent studies from India (Ramachandran et al., 2008) and China (Chan et al., 2009; Ramachandran et al., 2010, Ma et al., 2013) have shown greater rates of increase in diabetes prevalence in rural than in urban areas. Hwang et al. (Hwang et al., 2012) reported that across multiple surveys, there was evidence of a fivefold rise in the prevalence of diabetes from 1985 to 2010 in rural populations of developing countries.

Diabetes in the Diaspora

Population-based studies indicate a higher prevalence of diabetes among the South Asian diaspora compared with other ethnic and the local populations in many Western nations, including the U.S. and the U.K. (Misra et al., 2014; Lee et al., 2011; King et al., 2012 Karter et al., 2012; Kanaya et al., 2014; Bhopal et al., 1999). Among South Asians, T2DM is usually diagnosed at an earlier age and is associated with increased mortality compared with the white population in these countries.

1.17 Diabetes in Bangladesh perspective

5 million people have diabetes in Bangladesh (IDF SEA, 2013). About 22.7 percents patients are sufferings from T1D or insulin-dependent diabetes and 77.3 percents are suffering from T2D or non-insulin-dependent diabetes in Rajshahi city (Rahman et al., 2008). Diabetes mellitus particularly type 2 diabetes is now recognized as a major chronic public health problem in Bangladesh. The magnitude of diabetes remains unknown due to lack of countrywide survey. More than 80% of country population lives in rural areas but some studies showed that the prevalence is higher in urban areas. However, some small scale survey conducted by (Mahtab et al., 1983; Sayeed et al., 1995) showed that the prevalence of DM in the age group >15 years varied from 1.0 to 1.5% in a urban areas and 0.5 to 1.0% in rural areas. Sayeed et al. in 1997 conducted a study in rural Bangladesh and found the prevalence of type 2 diabetes was 2.1% (male 3.1% and female 1.3%). Age adjusted (30-64 years of age) prevalence was 2.23%. Recently, another study in 1997 among the rural, urban and sub urban population of Bangladesh showed that the combine prevalence among the rural and urban population was 5.2% of which rural prevalence was 3.8% and urban prevalence was 7.8%. Age adjusted (30-64 years) prevalence urban 8.0% and rural 3.8% (Sayeed et al., 1997). Although there was no large-scale national survey in Bangladesh, several small-scale surveys at intervals have been done over several years. Diabetes registry in BIRDEM, a referral center, also showed an increasing trend. Only 389 diabetic subjects were registered throughout the year 1960. This figure increased to 1181, 2363, 9641 and 17163 in the year 1970, '80, '90 and 2000, respectively. All these figures indicate that the magnitude of health problems related to diabetes in Bangladesh has been

increasing rapidly. It has been observed that the prevalence of diabetes was significantly higher in the urban than rural community of Bangladesh. So it is likely that the prevalence has been increasing with increasing urbanization. It has also been observed that the complications of diabetes were more frequent among the rural than urban and in poor than rich diabetic population.

In Bangladesh the literacy rate among women with diabetes in rural areas is low. Statistics available at BIRDEM for January 2002 indicated that 50% of rural women with diabetes were illiterate and only 34% had completed primary schooling. Their awareness and understanding of diabetes was poor (Mahtab et al., 2002).

Although the exact causes or risk factors are not known for increased mortality in Bangladeshis, both genetic predisposition and environmental factors are commonly attributed irrespective of ethnicity.

Women from different ethnic groups or pregnant women are more susceptible to risk of diabetes and/or adverse complications than others. The prevalence of diagnosed diabetes is much higher for Pakistani, Bangladeshi, and African Caribbean women than for women in the general population (European Institute of Women's Health, 2006).

Diabetes has a very significant cause of social, psychological and financial burdens in populations worldwide. Quality of lifestyle and health care system can also accelerate diabetes (Islam et al., 2013).

1.8 Rationality of the study

T2D is a serious complex disease caused by several environmental and genetic factors. It is one of most common diseases, requires life-long treatment, and is associated with increased mortality, mainly due to complications that occur later in life. More than three decades of genetic studies have identified several genetic disease variants and a longer list of putative associated genetic loci. These findings have greatly increased our understanding of the genetic background of T2D and have encouraged the development of genetic tools for mapping complex diseases. In our country there is no study of major genetic polymorphisms involved in the diabetes. Therefore, aim of this study is to identify

the association between T2DM and *TCF7L2* genetic variation among the Bangladeshi population and look at those are still to be detected in Bangladeshi population. This study may help our physician to understand the genetic risk factors and etiology of Bangladeshi diabetic population. This also may contribute in making plan and budget allocation to cope non-communicable epidemic disease T2DM.

1.19 General objective

As there is no previous genetic study regarding the *TCF7L2* variants on T2D risk in Bangladeshi population, so we aimed to investigate the association of *TCF7L2* genetic variants in diabetic patients.

Specific objectives

1. Investigate the association of *TCF7L2* genetic variation on T2DM patients in the northern region of Bangladesh,
2. Improve existing HRM method, and
3. Analysis of demographic characteristics in correlation with *TCF7L2* genetic polymorphism.

Chapter 2

Materials and Methods

For identification of *TCF7L2* genetic variants, blood of T2D patients and non-diabetic person (with no diabetes in family history) were used as sample. Sample of non-diabetic individuals served as control. However, after isolation of DNA from blood, various steps were followed to subject DNA to HRM method of real-time PCR.

2.1 Recruitment of the subject

This study included 657 Bangladeshi individuals, including 327 T2D patients were recruited from Rajshahi who came to check their diabetic status. After taking brief history, preliminary selection was done, and the purpose of the study was explained in details to them. Finally, their verbal consent was taken. Blood samples of non-diabetic person (n = 330) who have no diabetic in family history were one of the criteria in wild type selection.

2.2 Sample collection and biochemical test:

Overnight fasting (8-12 hours) blood was collected. Peripheral blood samples were collected under the ethical clearance of Institutional Biosafety and Ethical committee. Venous blood (3 ml) was obtained by vein puncture. In this regard consent of the individuals was collected for both DM type 2 patients and control non diabetic patients. Samples were randomly collected in EDTA K3 vacuumed tube. After collection, blood samples were stored at 4°C.Part of the blood was used for genomic DNA isolation and the rest used for selected blood parameter testing i.e. HbA1c, TG and Cholesterol.

Figure 2.1: Collected blood sample from T2D patients and non-diabetic individuals.

2.3 Isolation of genomic DNA from collected blood

Blood is a specialized body fluid composed of cells suspended in a liquid called blood plasma. Whole blood contains three types of cells: Red Blood Cells (RBCs), White Blood Cells (WBCs) and platelets.

RBCs do not have any DNA, as they lose their nuclei during maturation. The white blood cell component of the blood contains the DNA. Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Jena Bioscience, 2012) according to the manufactory instructions.

2.3.1 Procedure of genomic DNA isolation

Here Genomic DNA was isolated using manual of DNA purification from whole blood of Jena Bioscience. Towards this process, fresh whole blood of 300 µl from vacuum tube was taken in a 1.5 ml centrifuge tube. It was ensured that the blood sample was at room temperature (15-25°C) before beginning the protocol.

At first, 900 µl RBC lysis buffer was added and well-mixed by inverting the tube 6-8 times. Then the tube was incubated at room temperature for 3 minutes. The tube contents were mixed intermittently by inverting 2-3 times during incubation. The tube was centrifuged at 15000 rcf for 30 sec at room temperature. Supernatant was discarded carefully without disturbing the white blood cells such that very small amount (10-20 µl) of residue liquid remains back in the tube. The tube was vortexed vigorously so as to resuspend the white blood cells completely. Cell lysis buffer of 300 µl was added to the resuspend white blood cells. Then it was mixed gently by inverting 3-4 times to lyses the cells. If any cell clumps were present, the solution was incubated at 65°C (for 10 min) until the clumps were dissolved. Then the solution was vortexed.

Protein precipitation solution of 100 µl was added to the cell lysate and quickly mixed by vortexing for 30 sec. Then this mixture was incubated on ice for 5 min. After that, it was vortexed. Some protein clumps was visible after vortexing. Then this mixture was centrifuged at 15000 rcf for 1 min.

Above supernatant of the tube was transferred to a new 1.5 ml centrifuge tube. Isopropanol of 300 µl was added to that supernatant and mixed by inverting the tube gently. Then 1 µl glycogen was added and mixed gently by inverting the tube (20-30 times) till DNA in fibrous white fibrous form was visible. Centrifuge was done at 15000 rcf for 1 min at room temperature. Small white pellet of DNA was visible.

Supernatant was discarded without disturbing the white pellet. 300 µl washing buffer was added to that tube and mixed gently. Centrifuge was performed at 15000 rcf for 1min. Supernatant was discarded by pipetteing and residual supernatant was discarded by inverting the tube on a clean tissue paper without disturbing the white pellet. Then the tube was subjected to air dry for 20-30 min. DNA hydration solution of 50 µl was added and incubated at 65°C for 30 min to dissolve the DNA completely. Finally, this DNA was stored at -20°C.

2.4 Agarose gel electrophoresis

Gel electrophoresis is the standard lab procedure for separating DNA on the basis of size (e.g. length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA toward a positive electrode through an agarose gel matrix. Molecular biology research laboratory routinely uses agarose gel electrophoresis technique for observation and analysis of DNA. The gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. Individual DNA molecules undergoing agarose gel electrophoresis were viewed with the aid of a fluorescence microscope (Smith et al., 1989). For observation of genomic DNA, 0.8% agarose gel was used. Agarose gel electrophoresis was performed according to the protocol of Lee et al. (2012).

2.4.1 Preparation of agarose gel

1.5% agarose gel was prepared by melting 0.75 gm agarose in 50 ml of diluted TAE/TBE Buffer using a microwave oven. The melted agarose was allowed to cool to about 50ºC and 3 µl ethidium bromide was mixed and shacked and was poured into gel tray and combs were placed. After solidification of the gel, the comb was removed. During electrophoresis,

the gel was placed in a Horizontal electrophoresis apparatus containing TAE/TBE buffer and ethidium bromide.

2.4.2 Loading and electrophoresis of the sample

5 µl of DNA product was mixed with 2.0 µl of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. Electrophoresis was carried out at 100 volts for 35 minutes.

2.4.3 Visualization of the gel

The DNA products of the study samples were visualized by trans-illuminator. And the gel was photographed by a digital camera and transferred data to computer for further documentation.

2.5 DNA precipitation/purification with Phenol-Chloroform

We took 200 µl template/DNA with distilled water and added 100 µl $CHCl_3$. Then slowly added 100 µl Phenol (Vortex). Centrifuge at 13000 rpm for 5 minute. Collect the supernatant (about 200 µl) in an eppendorf tube. After that we added 20 µl CH_3COONa + 550 µl Ethanol + 3 µl Glycogen and placed it at -10°C for 30 minute and Centrifuge at 13000 rpm for 10 minute. Remove the supernatant, wash with 70% ethanol and dry and diluted with distilled water. Store at -20°C.

2.6 Measurement of optical density (OD) of isolated DNA

Optical density (OD) of DNA is generally determined by ultraviolet spectrophotometric measurement of the absorption at 260 nm on a sample of the DNA in solution. Nucleic acids, depending on base composition, absorb maximally at about 260 nm. Typically, quartz cuvettes are used to hold the samples as they do not absorb in the UV range the way most glasses and plastics. TE buffer or dH_2O is used to zero the instrument ('set reference') (Barbas et al., 2007) and the DNA samples themselves should be appropriately diluted in TE buffer or dH_2O such that the OD260 readings obtained are above ~ 0.02 (to avoid statistical error) but below ~ 0.8 (Beer's Law). OD of DNA was measured by using the protocol of Barbas et al. (2007).

2.7 Determination of concentration of isolated DNA from OD

Concentration of isolated DNA was determined with the help of software “Endmemo”. By putting the value of OD of isolated DNA to OD_{260} nucleotide concentration calculator of that website, Concentration of isolated DNA was determined when the light wave was 260 nm, the absorbance of light and concentration of DNA was calculated as:

$$C = A / (e * I)$$

Where,

C = concentration (µg/ml) of nucleic acid

A = absorbance (OD_{260})

I = width (cm) of cuvette; usually 1 cm stands for extinction coefficient of DNA which is follows, 1 OD_{260} Unit =50 µg/ml for double stranded DNA.

2.8 Template dilution series

For HRM, concentration of DNA has to be equal. If there is minute amount of DNA, it will be amplified with real-time PCR. Initially, template dilution series were carried out by 100 ng/ µl. From here, 3 µl DNA for each sample was mixed with 2 µl DNA loading buffer on a parafilm. Then this DNA was run on 0.8% agarose gel electrophoresis. After 10 minutes of loading, gel casting tray was placed to UV transillumination to observe DNA band. When there was an equal amount of DNA band, it had been understand that template dilution was successful.

2.9 Determination of optimum annealing temperature using gradient PCR

Running a gradient PCR for new primer sets helps to identify a single annealing temperature that will provide efficient and specific amplification of all targets (Clementi et al., 1993). The use of temperature gradient makes it possible to visualize the functionality of primer in simultaneously performed PCR amplification with increasing annealing temperature. An additional advantage of the method is that primer length can be reduced far below 47 nucleotides, as described by Shuldiner et al. (1991). Weitkamp et al. (2001)

have used gradient PCR to improve the efficiency and reliability of RT-PCR using tag-extended RT primers and temperature gradient PCR.

Gradient PCR is a technique that allows the empirical determination of an optimal annealing temperature using the least number of steps. This optimization can often be achieved in one experiment. The Agilent Technologies (Sure Cycler 8800) provides a gradient function that in one single run evaluates up to 12 different annealing, elongation, or denaturation temperatures. During the same run a number of possible concentration parameters can also be tested, row by row. Generally, block temperature is different from one another.

Non-specific secondary bands may form after the PCR reaction, which hinder, or even prevent, further analysis (cycle sequencing, mutation detection, etc.) or an unequivocal assessment of the PCR result. In such cases, PCR conditions must be optimized. This is normally achieved by titrating the magnesium, template, primer, dNTPs concentration and Taq-Polymerase concentration, "Hot Start PCR," "Touch-down PCR," adding detergents, reducing the PCR cycles or by gradually increasing the annealing temperature (Ferre et al., 1992).

The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. In most cases, this temperature must be empirically tested. The PCR is normally started at 5°C below the calculated temperature of the primer melting point (T_m).

2.9.1 Procedure of gradient PCR

All reaction components were assembled on ice in an eppendorf tube. Components were taken for 12 samples. Then it was mixed gently by quick spin (HERMLE, Labortechnik Gmbt, German).

Table 2.1: Components for PCR reaction mixture preparation

Component	Amount (50 µl reaction)
GoTaq® Hot start PCR master mix (2x)	25µl
10 µM Forward Primer	1µl
10 µM Reverse Primer	1µl
Template DNA	1µl
Nuclease-free water	22µl

PCR mixture of 50 µl from eppendorf tube was taken in PCR tube for each sample. PCR tubes were transfered from ice to a PCR machine with the block preheated to 95°C and thermocycling was begun.

Table 2.2: Thermal cycling conditions of PCR

<table>
<tr><th>Steps</th><th>Temperature (°C)</th><th colspan="2">Time</th></tr>
<tr><td>Hot start</td><td>95</td><td colspan="2">30 seconds</td></tr>
<tr><td>Denaturation</td><td>95</td><td>30 seconds</td><td rowspan="3">Repeated for 40 cycles</td></tr>
<tr><td>Annealing</td><td>55-62</td><td>30 seconds</td></tr>
<tr><td>Extension</td><td>72</td><td>30 seconds</td></tr>
<tr><td>Elongation</td><td>72</td><td colspan="2">10 minutes</td></tr>
<tr><td>Hold</td><td>4</td><td colspan="2">Infinite</td></tr>
</table>

2.10 Gel-electrophoresis of PCR products

Agarose gels act as a matrix which separate DNA based on size. Smaller fragments move faster through the gel and will appear towards the bottom of the gel or leading edge of the dye front.

1.5% agarose gels can separate DNA fragments from 0.2-4 Kb in size. By increasing the agarose to 3-4%, it is possible to get better resolution of smaller DNA fragments. The gels are stained with ethidium bromide to see the DNA fragments under UV light. Ethidium bromide is an intercalating dye (and a powerful mutagen) which binds to the DNA and fluoresces under UV light.

1.5% agarose gel electrophoresis was performed by following the procedure of Lee et al. (2012). However, 0.8% agarose gel electrophoresis was done for genomic DNA (section 3.8) according to this procedure.

2.10.1 Observation of PCR product

The DNA bands were visualized using UV transilluminator. The best conditions are found in well 10 (of the cycler) where the temperature was 61°C. The calculated primer annealing temperature was 56.5°C; the actual annealing temperature is 61°C. By using the gradient function of the universal block, a gradient of 52.1 to 65°C was set. The following test parameters were selected: denaturation 30s, annealing 30s, elongation 8s, Taq-Polymerase 0.75 units; duration of entire experiment.

2.11 Confirmation of optimization of TCF7L2 PCR conditions

For confirmation of optimization of PCR conditions for TCF7L2, PCR was also run again in the conditions in which DNA amplification was appropriate. The following test parameters were used: denaturation 95°C,10 s; annealing 60°C, 15 s; elongation 72°C, 20 s; Taq-Polymerase 0.75 units. After PCR of TCF7L2 gene, PCR product was run on 1.5% agarose gel and visualized under UV transilluminator.

In this experiment, the universal block was set to a uniform temperature in the annealing phase. The outstanding temperature homogeneity of the block ensures reproducible PCR results.

2.12 Mutation detection by HRM using real-time PCR and Sequencing

Quantitative or real time PCR (qPCR) is standard PCR with the advantage of detecting the amount of DNA formed after each cycle with either fluorescent dyes or fluorescently-tagged oligonucleotide probes. Q-PCR results can be obtained faster and with less variability than standard PCR due to sensitive fluorescent chemistry and elimination of post-PCR detection procedures.

Detection of mutation through real-time PCR is developed on the basis of HRM. High-resolution melting technology was initially developed for genotyping studies and is based on the comparison of the melting profiles of sequences that differ in base composition and has been shown to have a sensitivity superior to direct sequencing. Each double-stranded DNA molecule is characterized by a melting temperature or, for longer molecules, a melting profile. There is ample evidence that HRM have been used in mutation (Simi et al., 2008; Nomoto et al., 2006; Krypuy et al., 2006 and Do et al., 2008) and polymorphism (Guan et al., 2010 and Gan et al., 2010) detection. Researchers have developed several methods of quantitative PCR. One approach measures PCR product quantity in the log phase of the reaction before the plateau. This method requires that each sample has equal input amounts of nucleic acid and that each sample under analysis amplifies with identical efficiency up to the point of quantitative analysis (Kellogg et al., 1990 and Pang et al., 1990).

Real-time PCR with fluorscein-labeled oligonucleotide probe has been also used to detect mutation by monitoring Tm values of sequences (Crockett et al., 2001).

According to the Eco™ real-time PCR system user guide, the melting temperature of a PCR product is investigated by subjecting it to an increasing temperature gradient in the presence of a DNA binding dye, which emits fluorescence when bound (intercalated) to double-stranded DNA. The fluorescent dye will bind to double-stranded DNA emitting high levels of fluorescence until the temperature reaches the melting temperature of the

PCR product. At the melting temperature, the PCR product dissociates into two single strands and the dye can no longer bind and fluoresce, and a sharp drop in the fluorescence is observed. The changes of fluorescence levels across a denaturing gradient describe an amplicon's melting profile. Melting temperature is different for mutated DNA from normal DNA. Furthermore, heterozygous and homozygous mutation can also be detected by melting profile. Mutation detection of diabetic and non diabetic person was done by following the method of Eco™ real-time PCR system user guide. For real-time PCR in this study, GoTaq® qPCr master mix technical manual (Promega) and CXR (carboxy-X-rhodamine reference dye) were used.

2.12.1 Preparation of the reaction mix of real-time PCR for experiment

Components, for experiment, were taken in a conditions described in table 2.3

Table 2.3: Reaction condition of real-time PCR for 50 samples

Component	Volume (µl) (10 µl reaction)	Sample number	Amount (µl)
GoTaq® qPCR master mix (2x)	5	50	250
Nuclease free water	3		150
Upstream primer	0.5		25
Downstream primer	0.5		25
Template	1	For each sample	

2.12.2. Procedure of real-time PCR

Real-time PCR mixture was allowed to thaw. Net book PC and ECO was turned on and waited until the ECO Ready light is solid blue. It was need to be confirmed that block and optical path are clear of visible contaminants and there is no physical damage to the system, such as dents, frayed cords and damaged levers. A 48 well plate was placed in to

Eco sample loading dock, alingning the notch with the matching indentation on the adapter.

The dock light was turned on and the dock was inclined to a comfortable angle for pipetting. Samples and qPCR reagents were pipetted in to the plate. The plate was sealed with Eco optical seal. The plate was held on the Eco sample loading dock, drag the squeegee firmly across the surface to ensure the seal is secured. Eco lid was opened and the plate was placed on the block, aligning the notch against the left corner. The lid was closed. The heated lid automatically created a seal around and on top of the plate to prevent evaporation.

Software was opened by double click Eco Icon on the desktop. Application option was selected to run High Resolution Melting experiment. Experiment name of up to 20 characters was entered. Thermal profile was adapted according to the experiment (Figure 2.2).

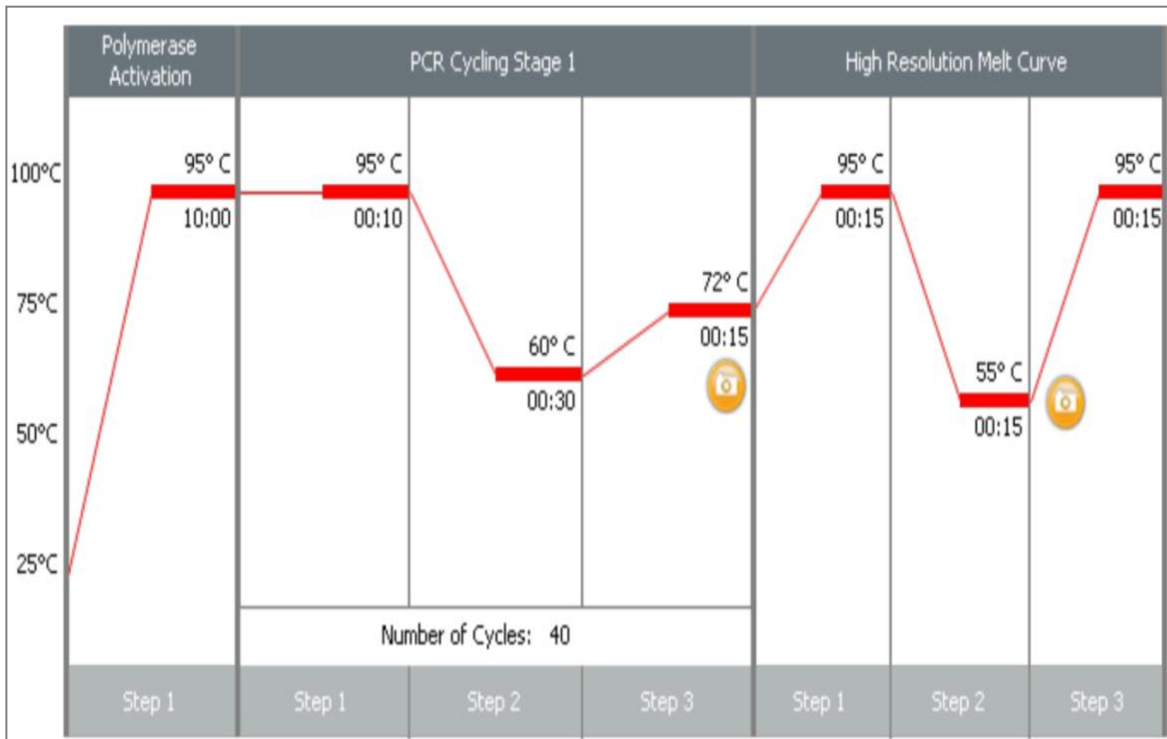


Figure 2.2: Thermal profile for real-time PCR.

2.13 Demographic characteristics analysis

2.13.1 Body Mass Index (BMI) calculation

The body mass index (BMI) is a value derived from the mass (weight) and height of an individual. BMI provides a simple numeric measure of a person's *thickness* or *thinness*, allowing health professionals to discuss weight problems more objectively with their patients. BMI was designed to be used as a simple means of classifying average sedentary (physically inactive) populations, with an average body composition. For these individuals, the current value recommendations are as follow: a BMI from 18.5 up to 25 kg/m^2 may indicate optimal weight, a BMI lower than 18.5 suggests the person is underweight, a number from 25 up to 30 may indicate the person is overweight, and a number from 30 upwards suggests the person is obese. Lean athletes often have a high muscle to fat ratio and therefore a BMI that is misleadingly high relative to their body fat percentage.

The BMI is universally expressed in kg/m^2, resulting from mass in kilograms and height in meters. If pounds and inches are used, a conversion factor of 703 (kg/m^2)/(lb/in^2) must be applied. When the term BMI is used informally, the units are usually omitted.

$$\mathrm{BMI} = \frac{\mathrm{mass(kg)}}{(\mathrm{height(m)})^2}$$

$$= \frac{\mathrm{mass(lb)}}{(\mathrm{height(in)})^2} \times 703$$

2.13.2 Glycated haemoglobin (HbA1c), Triglycerides (Tg) and Cholesterol level analysis

Glycated haemoglobin (HbA1c)

The term HbA1c refers to glycated haemoglobin. It develops when haemoglobin, a protein within red blood cells that carries oxygen throughout your body, joins with glucose in the blood, becoming 'glycated'. By measuring glycated haemoglobin (HbA1c), clinicians are able to get an overall picture of what our average blood sugar levels have been over a period of weeks/months. For people with diabetes this is important as the higher the HbA1c, the greater the risk of developing diabetes-related complications.

To measure HbA1c level, collected blood sample was taken, and used to produce a reading. In some cases, such as with HbA1c testing for children, a single droplet of blood may only be required to find out how much haemoglobin A1c is present.

Triglycerides (Tg)

Triglycerides are blood fats that are a flexible source of energy. The body can convert triglycerides into glucose and triglycerides can also be stored in adipose tissue (fat cells). The process of converting triglycerides into glucose is known as gluconeogenesis and is performed by the liver.

High triglyceride levels, also known as hypertriglyceridemia, are often the result of either an additional medical condition or having a high calorie diet. High triglyceride levels tend to be particularly common in patients with uncontrolled type 2 diabetes.

Cholesterol

Cholesterol is mainly comprised of fat and lipoproteins. A lipoprotein is comprised of cholesterol, protein, and fat (triglycerides). Cholesterol comes from two sources. Our body manufactures some cholesterol on its own. In addition, cholesterol comes from animal products, such as milk, eggs, cheese, and meats. Cholesterol has the consistency similar to gum or wax. Small amounts of cholesterol are important for a healthy cell membrane (good cholesterol), and some cholesterol has been deemed, “the bad cholesterol,” due to these cholesterol particles tends to cause atherosclerosis, or hardening of the arteries. Some cholesterol is “good,” cholesterol, that tends to carry the bad cholesterol away and out the body. That is why you want to have the good cholesterol around.

A little part of the blood was used for genomic DNA isolation and the rest was used for selected blood parameter testing *i.e.* HbA1c, Tg and Cholesterol. HbA1c, Tg and Cholesterol level was determined following standard procedure in a local authorized diagnostic center.

2.14 Statistical analysis

Statistical analysis was carried out using the statistical program SPSS (Version 19, IBM Corporation, USA). Frequencies of genotypic difference were compared using the χ^2 (Chi-square) test. One-way analysis of variance (ANOVA) was utilized to compare the clinical features among groups. Fisher's test was performed to check the correlation between age and genotypes.

Chapter 3

Results

High Resolution Melting (HRM) is a homogeneous and highly powerful method for SNP genotyping, mutation scanning and sequence scanning in DNA samples. This has been possible by the recently improved double-stranded DNA binding dyes as well as the next-generation real-time PCR instrumentation. Ultimately the HRM analysis generates melting (dissociation) curve which indicates the possible presence of sequence variation in its presentation.

TCF7L2 gene is well reported to be associated with type 2 diabetes. Precisely, rs7901695 polymorphism of *TCF7L2* gene has been previously reported to be associated with diabetes type 2 in several ethnic groups. However, there was no genetic analysis has been carried out to show the relationship of rs7901695 with type two diabetes in the Northern region of Bangladeshi population. Thus, in this study we have analyzed a total of 657 individuals, of which 330 were non-diabetic controls and 327 DM type 2 individuals. Here, persons with no record of diabetics as well as not even in the family was considered as non-diabetic control. Besides, young individuals within the control group could potentially interfere with association tests, due to the possibility that these individuals may develop the disease later in life. Therefore, we particularly selected the control group with mean age above 55 years. In rs7901695 genotype, purine is converted to pyrimidine. A and G both are purine and they are expected to be converted to either C or T in case of rs7901695. Sequence analysis of our control sample shows that A and G both are present in homozygous form (Figure 3.2 & 3.3). Once we performed our HRM analysis with these samples we found that persons who have no mutation in any allele are aligned with wild type curve. Moreover, generated curves are as it would have been for GG or AA allele (Figure 3.1).

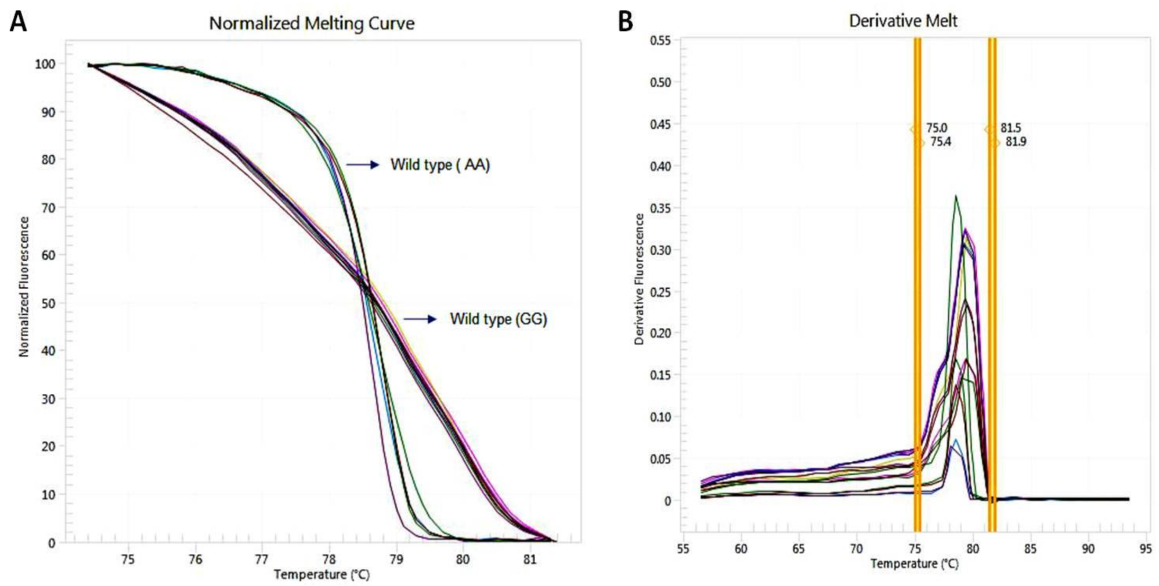


Figure 3.1: Normalized melting curve (A) and Derivative melting curve (B) in non-diabetic control sample (AA & GG).

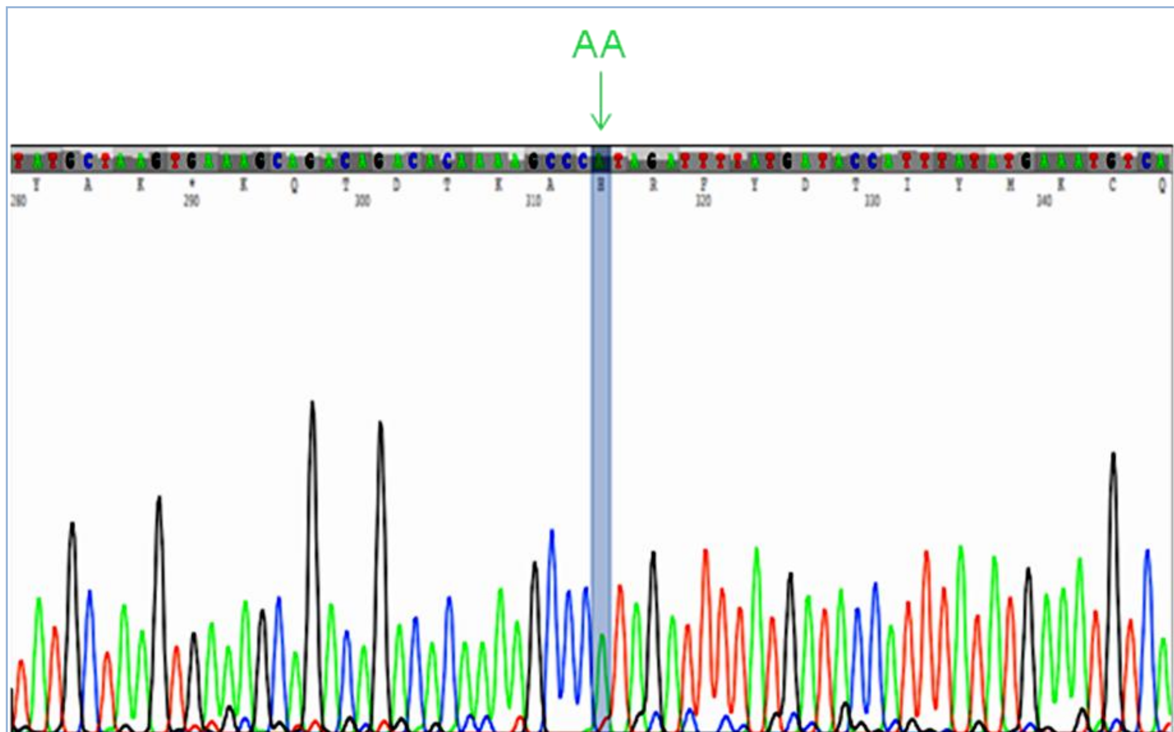


Figure 3.2: Sequencing result of non-diabetic control sample (AA).

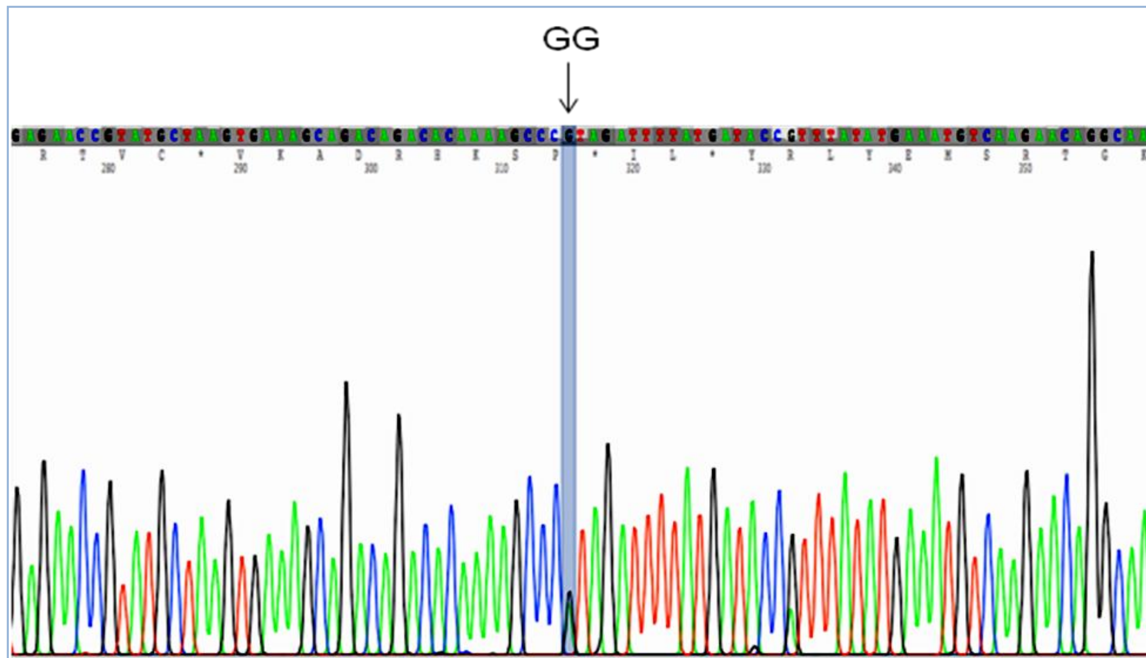


Figure 3.3: Sequencing result of non-diabetic control sample (GG).

3.1 Possible Combinations in rs7901695

Purine to pyrimidine conversion in rs7901695 generates multiple genotypes once both heterozygous and homozygous condition is considered. In these variants, we found 8 types of possible genotypes (Table 3.1).

Table 3.1: Different combination of genotypes due to purine to pyrimidine conversion in rs7901695

Allele	Wild type	Heterozygous	Homozygous
A ⇨ C	AA	AC	CC
A ⇨ T	AA	AT	TT
G ⇨ C	GG	GC	CC
G ⇨ T	GG	GT	TT

3.2 Improvement of HRM technique for rs7901695:

Hence, methods such as high-resolution melting (HRM) (Zhou et al., 2005; Erali et al., 2010) are commonly used in clinical applications for mutation detection (Kennerson et al., 2007; Margraf et al., 2006). HRM is normally conducted in a post-PCR fashion and can be easily done in a routine laboratory with a real-time PCR machine. However, PCR-HRM may often miss mutations less abundant than approximately 3%−10% (Ney et al., 2012) that may be clinically significant. Therefore, increasing detection sensitivity in HRM mutation scanning can increase its clinical value. Correct execution of a HRM genotyping experiment include type and quality of DNA material, template DNA preparation, primer and amplicon design, and pipetting consistencies, as well as physical limitations in melting curve distinction for alternative variants. In our initial experiments, we tried following the protocol by book but it was very difficult for us to differentiate the melting curve. The following graphs are illustrating our HRM results in which we often found overlying melting curves (Figure 3.4). So we wanted to improve our methodology.

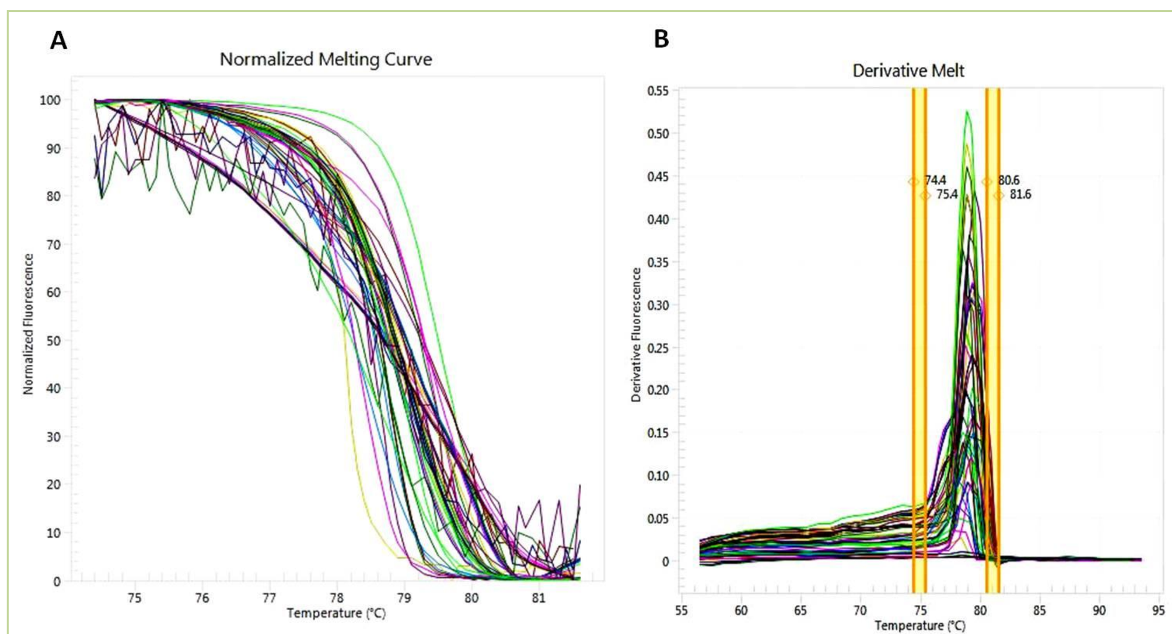


Figure 3.4: Normalized melt curve (A) and Derivative melt curve (B) before modification of methodology.

HRM analysis of GC rich sequence is often a challenge due to its high stringency of hydrogen bonds. However, both Formamide and DMSO were previously shown to improve thermal profile of DNA sample in similar manner (Song et al., 2015). Formamide is another chemical added in PCR and was expected to mimic the effect like DMSO. Thus, here we examined the effect of both by adding 7% DMSO and 0.2% Formamide to our HRM reaction. DMSO is used for increasing the amplification efficiency of GC-rich sequences (Jensen et al., 2010; Varadaraj et al., 1994), because it helps open up secondary structures and weaken hydrogen bonds between base pairs (Chester et al., 1993; Escara et al., 1980). Destabilization of DNA via addition of betaine before melting analysis of real-time PCR products generates a narrower melting peak for the probe–template duplex (Luo et al., 2011), whereas addition of high-salt buffer may improve clustering in HRM analysis (Vossen et al., 2009). Graziano et al., 2005 demonstrated that DMSO added before melting curve analysis with the no saturating dye SYBR Green I increases the separation between WT and mutant amplicons. Because HRM uses saturating dyes for high sensitivity and reproducibility (Wittwer et al., 2003; Monis et al., 2005), we anticipated that addition of DMSO would have an even more pronounced effect than SYBR Green I on the thermodynamic difference between mutant and WT amplicons. Once we added DMSO to make a final concentration of 7% and Formamide to make a final concentration of 0.2%, we found that both DMSO and Formamide increased that thermal profile of the amplicons. This finally created a physical separation of individual melting curves compared to non-treated HRM reaction (Figure 3.5). However, in terms of reproducibility and performance, we found Formamide is better than DMSO.

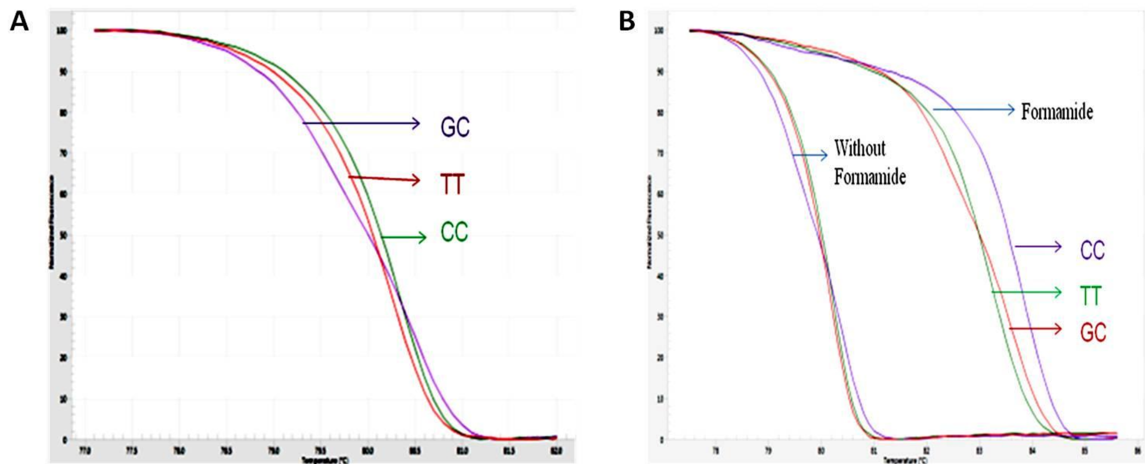


Figure 3.5: Normalized Melt Curve (A) Without DMSO and Formamide (B) Without Formamide and using Formamide.

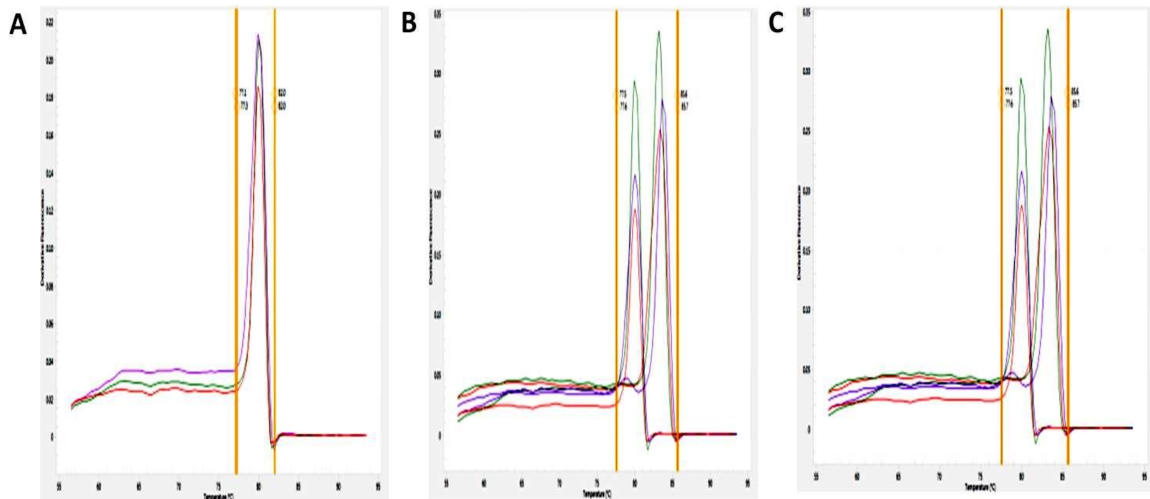


Figure 3.6: Derivative Melt Curve: (A) without DMSO and Formamide (B) using Formamide (C) using DMSO.

If we observe Figure 3.5 A, the curves were compact and hardly distinguishable (without DMSO and Formamide), but compare to the first one (Figure 3.5 A), the second one (Figure 3.5 B) is readily distinguishable (with Formamide). Further we followed this concentration of formamide in rest of HRM analysis of rs7901695.

3.3 HRM analysis of rs7901695:

An intrinsic feature of the HRM technique, where the shape of the melting curve is determined by the nucleotide composition of the amplicon. Besides, there will be a change in the centered thermal peak of the polymorphisms when compared with normal alleles. Here in this study we have analyzed the *TCF7L2* promoter region polymorphism rs7901695. In this regard our amplicon size was of 110 nucleotides long for HRM analysis. HRM analysis shows that the melt peak of the homozygote AA and GG genotype is centered at 78.8°C and 79.6°C respectively (Figure 3.7 A & B). Whereas the CC and TT allele's melt peak was centered at 79.4°C and 79.2°C (Figure 3.7 A). Heterozygote allele AC and GC melting peak was centered at 79.0°C and 79.2°C (Figure 3.7 B). In our HRM analysis we didn't find any GT and AT allele.

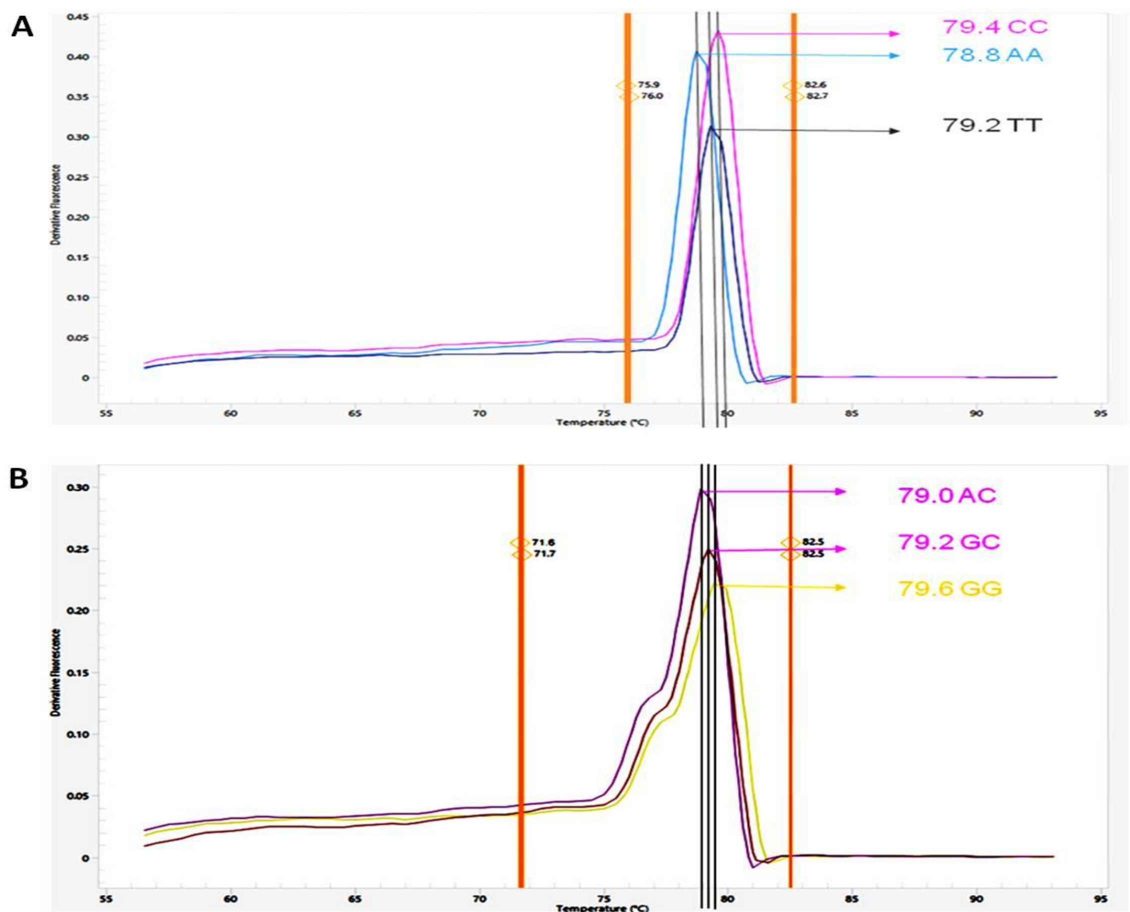


Figure 3.7: Derivative Melt Curve of (A) AA, CC and TT alleles, and (B) GG, GC and AC alleles.

3.4 HRM analysis of diabetic samples (327 samples)

In this research work, we have analyzed a total of 657 individuals, of which 327 DM type 2 individuals. Each time, HRM analysis was carried out in a 48 well PCR plate. Our melting curve shows that often 48 samples all together have a large spread and overlapping distribution of genotypes (Figure 3.8). Thus to increase our confidence, we excluded samples from plate layout in Eco Study software in post PCR analysis. This reduction or thinning of melt curve allowed us to compare each sample with assurance (Figure 3.9). Besides we have also compared the normalized carve with other generated reports as shown in Figure. 1.10 (You et al., 2013).

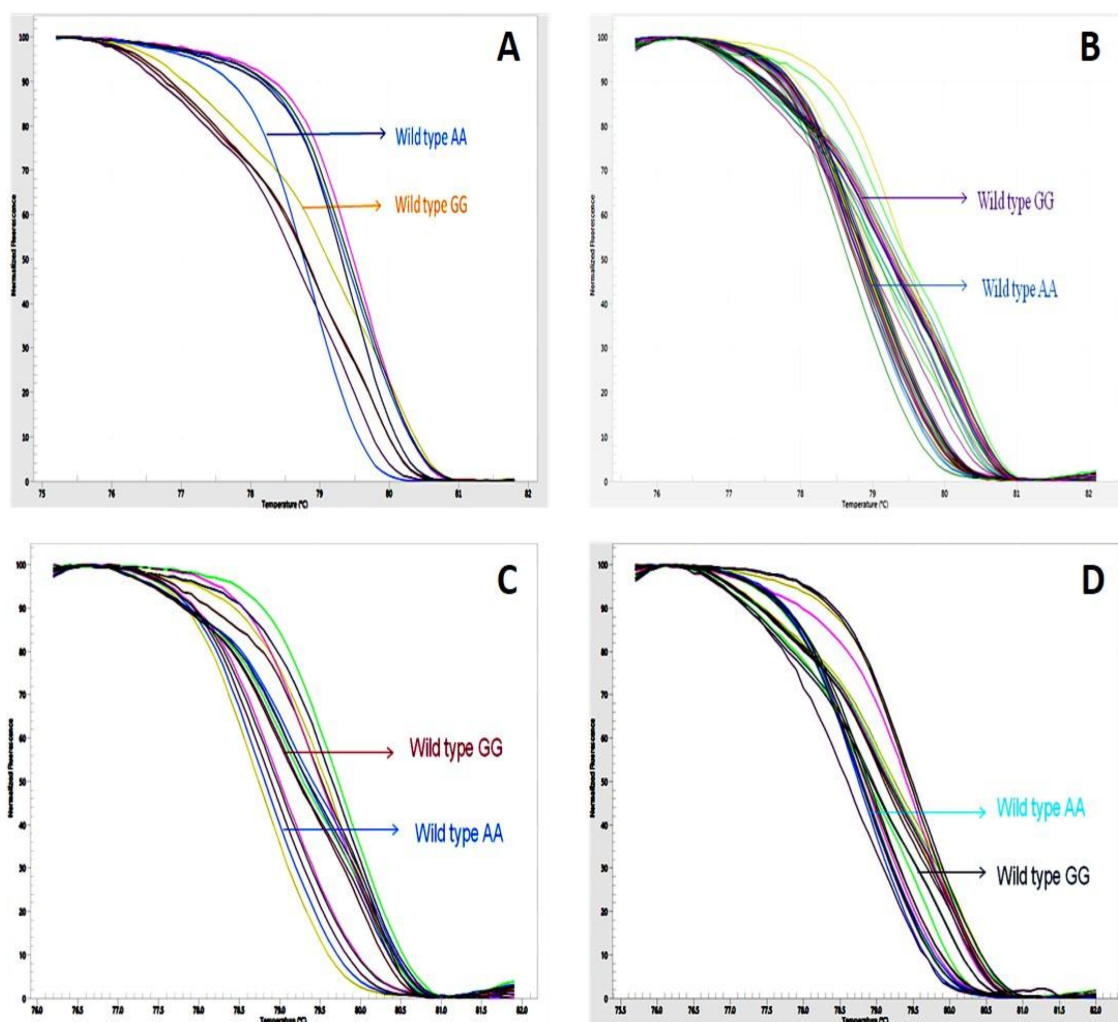


Figure 3.8: Normalized Melt Curve for diabetic (A) sample 1-12, (B) sample 13-55, (C) sample 56-75, (D) sample 76-100 (continued.)

Figure 3.8: Normalized Melt Curve for diabetic (E) sample 101-143, (F) sample 144-168 (G) sample 169-210, (H) sample 211-253, (I) sample 254-284, and (J) sample 285-327.

3.5 Examples of generated normalized curve

Our Illumina Eco qPCR machine has 48 wells. After every HRM PCR run generated overlapping normalized curve. Thus we systematically either excluded or included each well to better understand every generated curve. Finally, we have Identified 6 different types of genotypes in our HRM analysis (Figure 3.9).

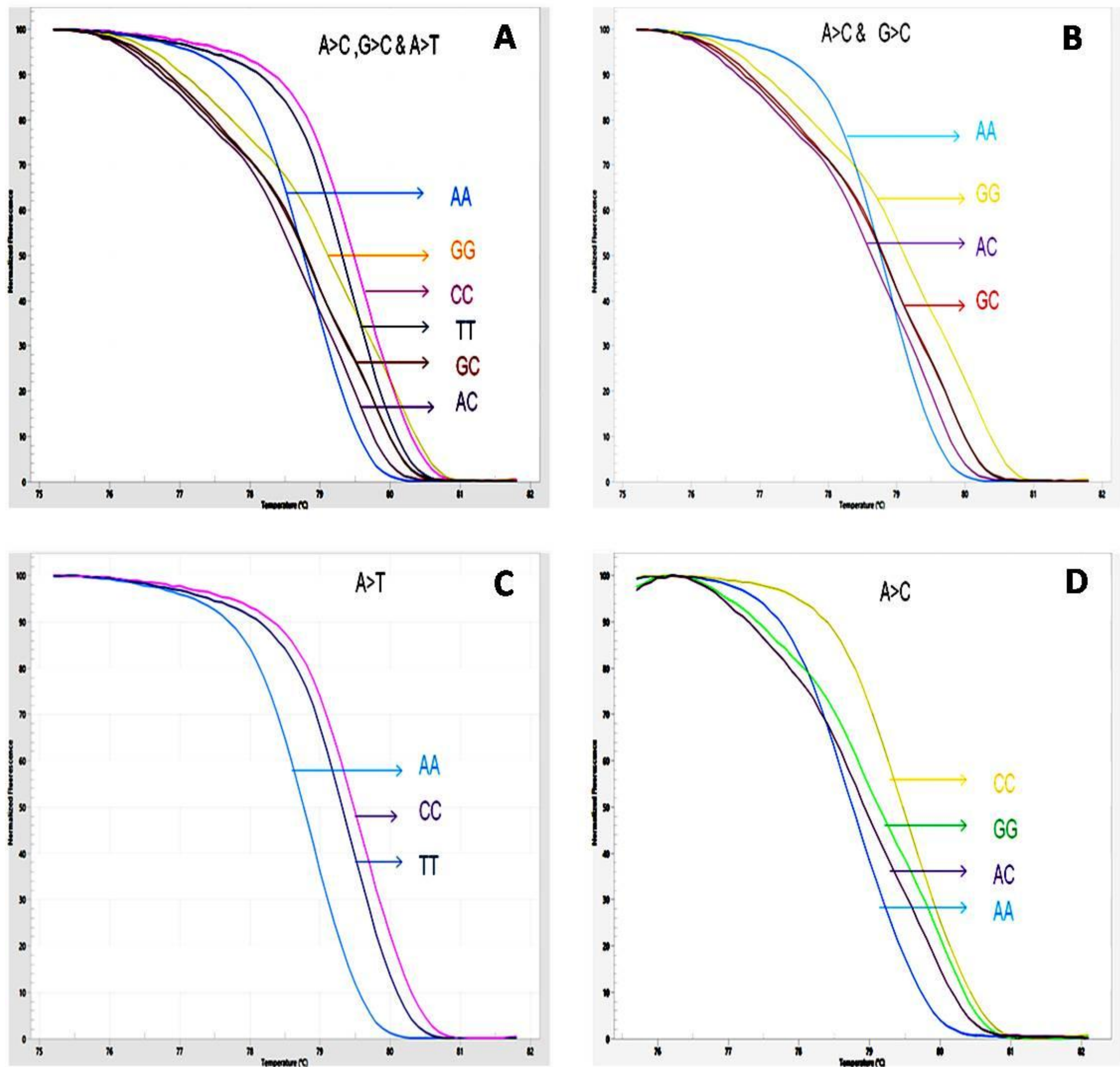


Figure 3.9: Examples of different normalized melting carve after thinning. Identified mutations (A) A>C, G>C, A>T (B) A>C, G>C (C) A>T (D) A>C are shown respectively.

3.6 HRM analysis of non diabetic patients (330 samples)

Here we have analyzed a total of 330 non-diabetic patients**.** In this case we didn't find any genotype other than GG and AA allele. (Figure 3.10) presents non-diabetic HRM melting curves.

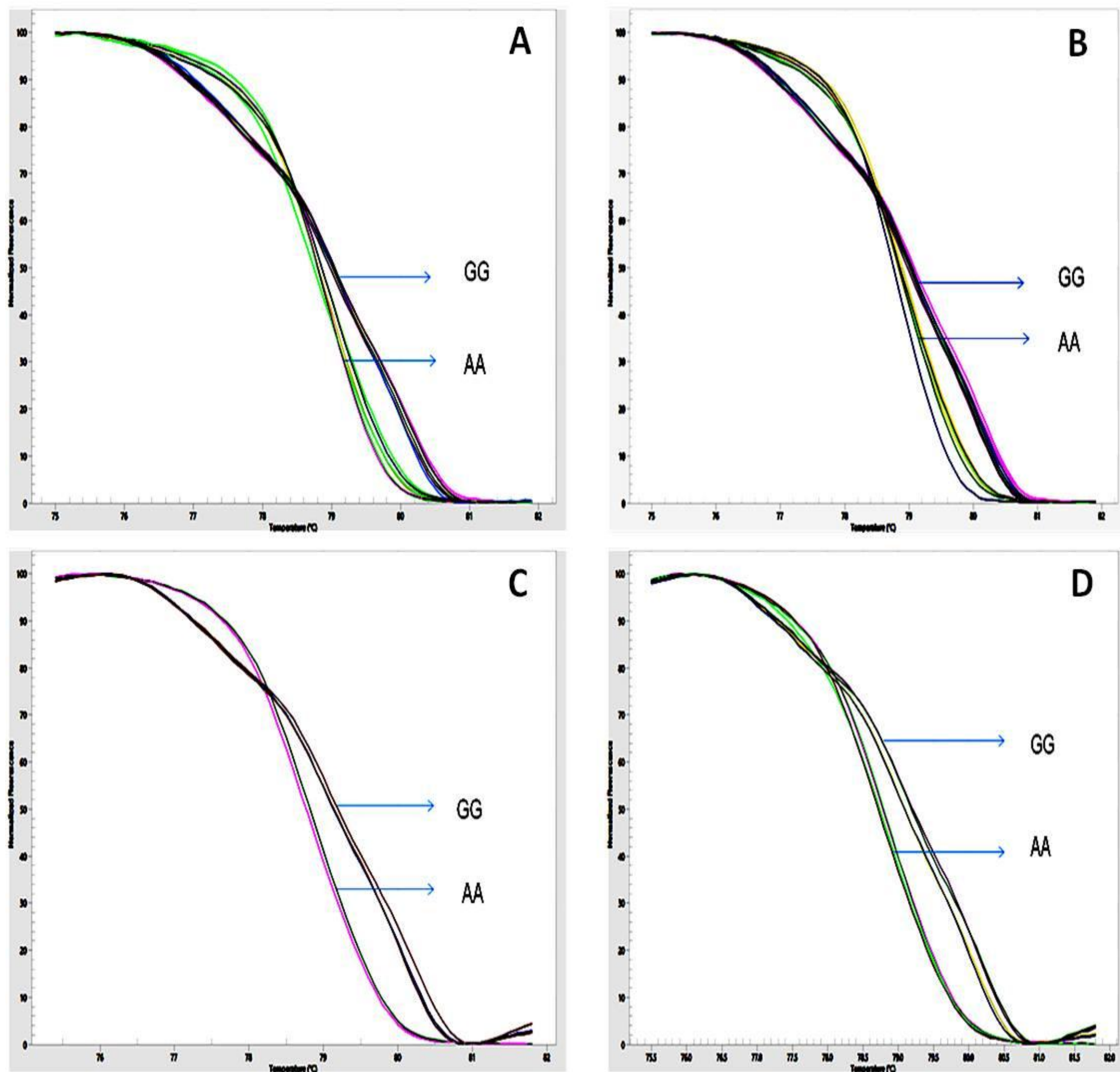


Figure 3.10: Normalized Melt Curve for non-diabetic (control) (A) sample 1-24, (B) sample 24-42, (C) sample 43-55, (D) sample 56-68, (continued..)

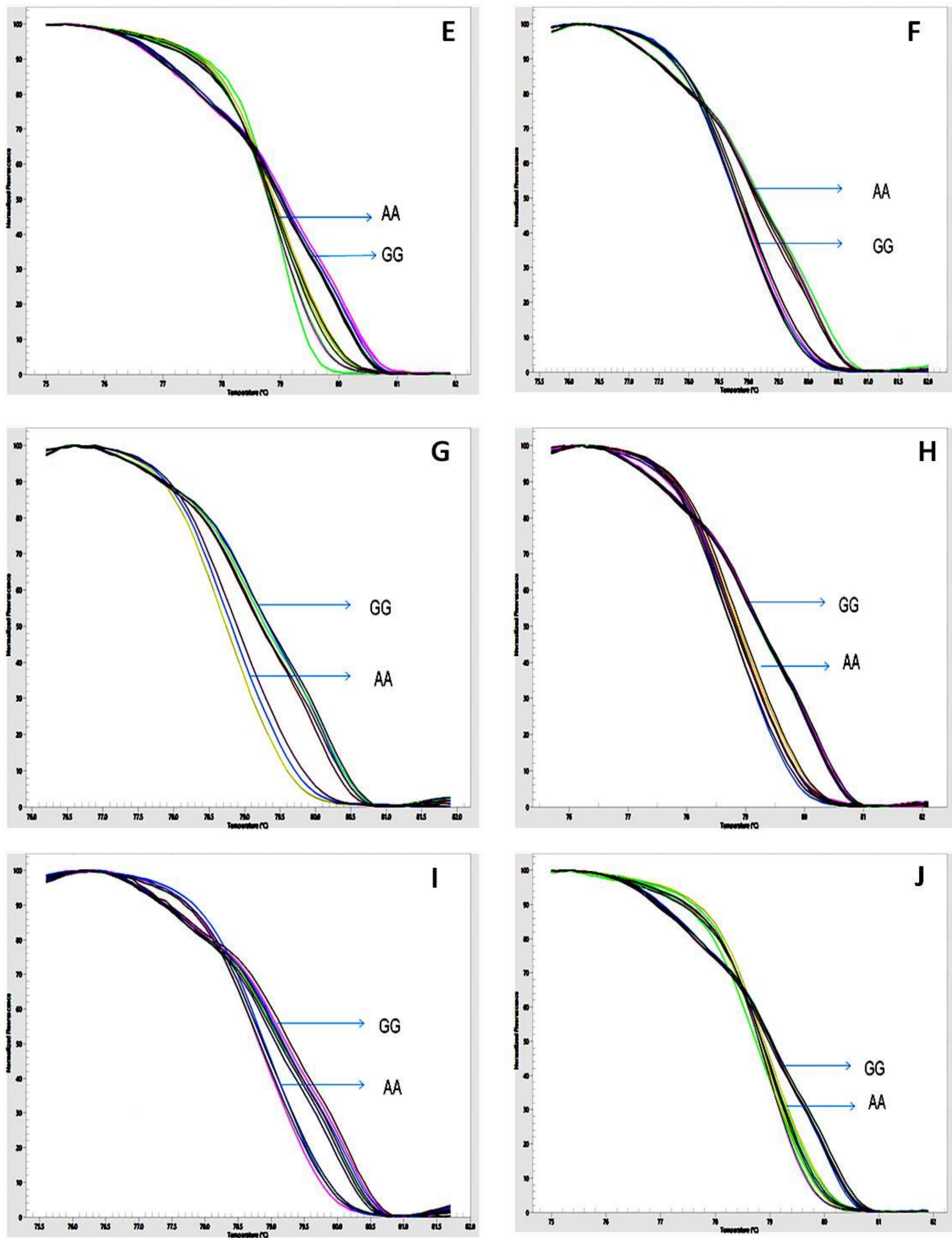


Figure 3.10: Normalized Melt Curve for non-diabetic (control) (E) sample 69-93, (F) sample 94-112, (G) sample 113-137, (H) sample 138-156, (I) sample 157-181, and (J) sample 182-200 (continued..)

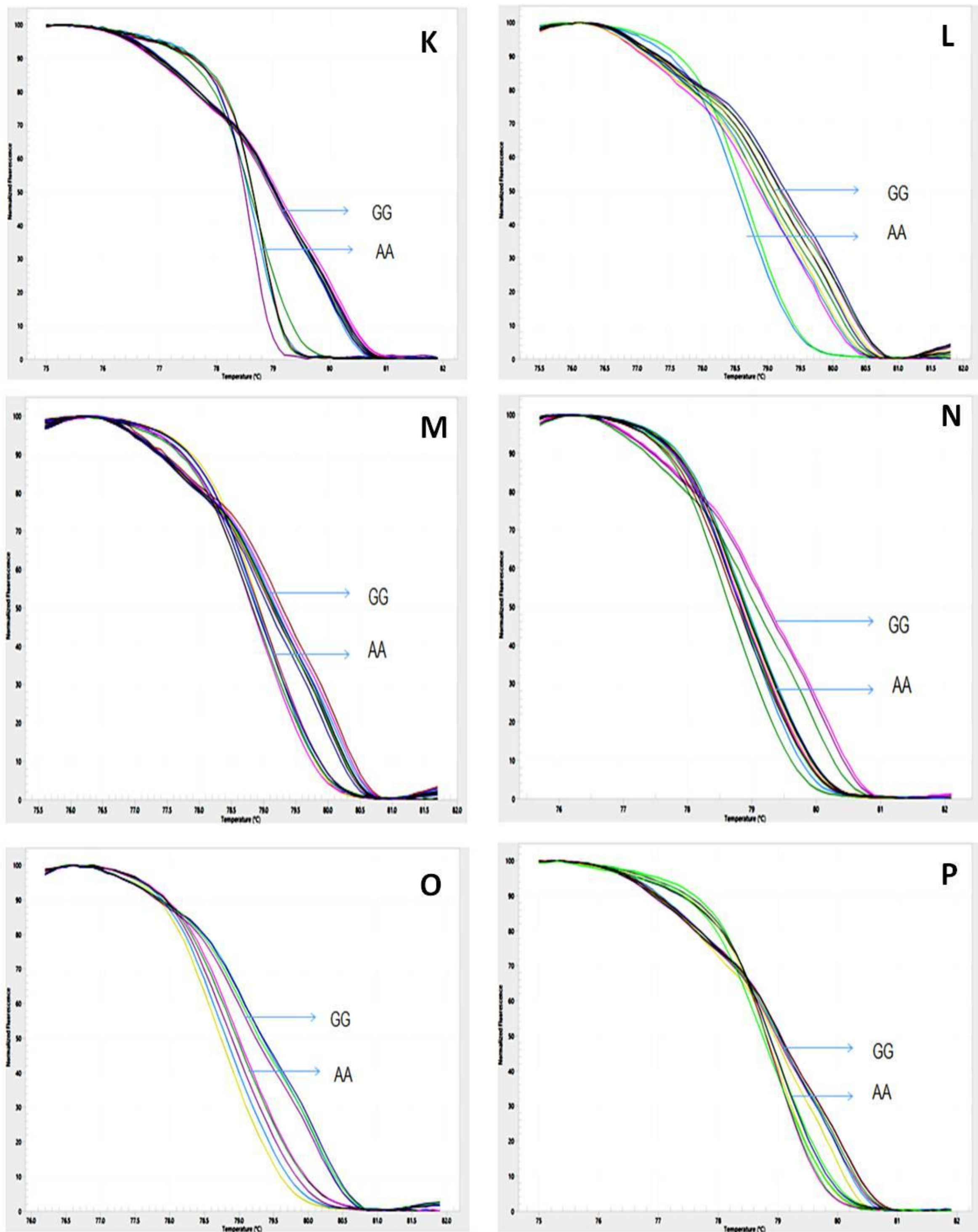


Figure 3.10: Normalized Melt Curve for non-diabetic (control) (K) sample 201-225, (L) sample 226-243, (M) sample 244-274, (N) sample 275-293, (O) sample 294-317, and (P) sample 318-330.

In this variants (rs7901695), we have found wild type AA in 139, GG in 101, heterozygous AC in 12 and GC in 23 and homozygous CC in 41 and TT in 11 among our total sample of 327 (Figure 3.9). The allele frequency of rs7901695 in the control group was within the Hardy-Weinberg equilibrium (0.05) at 95% confidence level and associated with DM type-2 (Table 3.2). However, we didn't find any genotype in 330 non diabetic patients other than GG and AA allele (Figure 3.10).

Table 3.2: Distribution of allele frequency of *TCF7L2* (rs7901695) polymorphism

Allele genotype	T2DM patients	Non-diabetic control	*P* value
GG	101 (30.89%)		
AA	139 (42.5%)		
AC	12 (3.67%)		
GC	23 (7.03%)	330	0.00482 (Significant)
CC	41 (12.5%)		
TT	11 (3.36%)		
Total	**327**	**330**	

In this research work, we have studied the association of DM type 2 with *TCF7L2* polymorphism in northern region of Bangladeshi population and their correlation with DM type 2 symptoms. We have also studied few anthropometric risk factors of DM type 2 in northern region of Bangladesh.

3.7 Demographic characteristics analysis

The anthropometrical, and biochemical characteristics of the participants are shown in Table 4.2. All characteristics of the participants significantly differed between T2DM and non-diabetic control. Our analysis shows that the means of BMI, HbA1c, and triglyceride (TG) and cholesterol among DM type 2 patients were higher than control

group. The control samples mean BMI were 28.85 ± 2.88 and 29.46 ± 2.57 for T2DM. Mean of HbA1c among diabetic individual (50.20 ± 8.84) is higher than non-diabetic controls (37.36 ± 2.61). The means of TG and Cholesterol between patients and non-diabetics are also higher (Table 3.3). Based on this table the significant difference was detected in the level of BMI (P value = 0.0049), HbA1c (P value = 0.0001), TG (P value = < 0.0001), and Cholesterol (P value = 0.0001) between T2DM and non-diabetic control (Table 3.3).

Table 3.3: Demographic characteristics analysis of T2D patient and non-diabetic control

Parameter	T2DM	Non-diabetic control	*P* value
BMI	29.46 ± 2.57	28.85 ± 2.88	0.004 significant
HbA1c (mol/L)	50.20 ± 8.84	37.36 ± 2.61	0.001 significant
Tg (mg/dL)	156.02 ± 27.93	145.45 ± 19.70	0.001 significant
Cholesterol (mg/dL)	228.92 ± 28.30	218.38 ± 20.19	0.001 significant

Chapter 4

Discussion

Type 2 diabetes (T2D) is a metabolic disorder that is associated with impaired insulin secretion. The processes concluding in impaired insulin secretion are not fully understood, but both genetic and environmental factors are thought to play a role. Studies in the recent past have observed a significant number of increase of the disease particularly in the developing country possibly due to changes in life style and food habit (Egger et al., 1997; Mehta et al., 2010). However, case-control studies often find the differences of allele frequency of genes between case and control patients that correlate to diseases. In this regard, Transcription Factor 7-Like 2 (*TCF7L2*) variants are the strongest known genetic risk factors for T2D (Grant et al., 2006). Though the gene was initially thought to play role only in cancer but later onset it was found to be involved with, T1D, T2D and gestational diabetes as well (Ali et al., 2013; Damcott et al., 2006). Primarily, a strong linkage signal was mapped for T2D susceptibility in chromosome 10q in a Mexican-American population; later fine mapping showed that risked locus is located in intron 3 of the *TCF7L2* gene. This locus showed to harbor five different variants i.e rs12255372, rs7903146, rs7901695, rs11196205, and rs7895340 (Grant et al., 2006). All these five *TCF7L2* variants have been associated with T2D susceptibility in multiple ethnic groups (Cauchi et al., 2006; Sale et al., 2007; Groves et al., 2006; Chang et al., 2007). Particularly, some variants increase the risk of T2D 1.5-fold in heterozygotes and 2.4-fold in homozygotes, corresponding to a population attributable risk of 21% (Grant et al., 2006).

Intronic distributions of the polymorphisms are expected to be seemingly harmless because of not being the part of the protein coding region. However, over recent years, the pathological alterations that can be directly linked with aberrant splicing processes have grown exponentially, and the study of the complex network interactions between defective splicing and occurrence of disease has become a central issue in the medical research field (Garcia-Blanco et al., 2004). *TCF7L2* has 17 exons, of which five are alternative spliced (Duval et al., 2000; Pang et al., 2013). Within these alternatively spliced isoforms longest version of the transcript is found in the beta cells, whereas the smallest version is available in the adipose tissue. This suggests that the *TCF7L2* has tissue specific splice variants and the gene is tightly controlled by a splicing mechanism. However, the correlation between the rs7901695 and its effect on splicing mechanism yet to be determined.

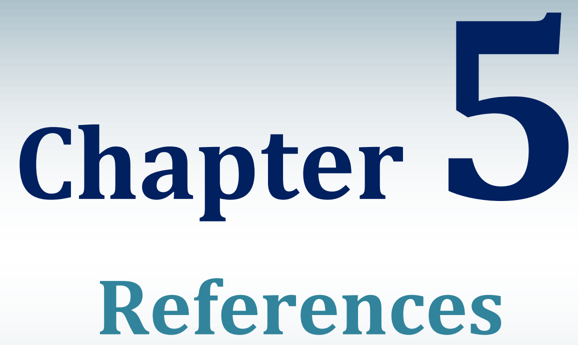
In this research work we have analyzed rs7901695 variants within the control and T2D. Like other four variants of the same intronic locus, rs7901695 is strongly associated with T2D. Previously, rs7901695 has been shown to be associated with T2D within Indian population (Humphries et al., 2006; Gupta et al., 2010; Sanghera et al., 2008). However, Indian population is widely distributed with a good number of different ethnic groups. *TCF7L2* association with T2D was carried out within Shikh ethnic group, north Indians or even in migrated Indian population in United Kingdom. In those studies, Bengali ethnic group was not included. This ethnic group is partially distributed in Calcutta of India and Bangladesh. In this research work, we focused on the Northern region of Bangladeshi population. Our findings shows that there are almost equal distribution of A/A and G/G allele in diabetic population as well as in control group. However, there was a significant association between variants and T2D patients. Our analysis showed that the attributed risk of TT allele is 3.36% and 12.5% for CC allele in diabetic patients. For heterozygote, this risk factor is 10.39%.

TCF7L2 is a major element of the WNT signaling pathway, and it is involved in the growth of a wide variety of cell lineages and organs (Klaus & Birchmeier, 2008). Possible mechanisms through which *TCF7L2* variants causes T2D include its role in adipogenesis, myogenesis, and pancreatic islet development, as well as in β-cell survival and insulin secretory granule function through WNT pathway (Shu et al., 2014; da Silva et al., 2009). Adipogenesis may ultimately lead to obesity, a major symptom of diabetic phenotype. Our analysis of clinical characteristics shows that BMI, Tg and cholesterol is significantly high is T2D patients over non diabetic patients. However, symptoms like decreased insulin secretion, glucose production, and glucose tolerance in case of *TCF7L2* variants are because of direct effects on pancreatic islet beta cells (Lyssenko et al., 2007; Schäfer et al., 2007). In fact, deregulation in glucose metabolism, reduced dispensation of proinsulin, and raised up levels of gastric inhibitory peptide and HbA1c can be observed in non-diabetic individuals with *TCF7L2* polymorphisms before the onset of T2D (Gjesing et al., 2011). Particularly for rs7901695 there is an increased proinsulin and proinsulin-to-insulin ratio. A meta-analysis revealed that the T-genotype of the *TCF7L2* rs7903146 is a significant risk factor for impaired proinsulin conversion which ultimately leads to T2D (Shen et al.,

2015). Besides, *TCF7L2* plays a role in transcription of the glucagon-like peptides GLP-1 and GLP-2. These peptides play a role in postprandial insulin secretion. GLP-1 sustains glucose homeostasis through the biological actions like induction of insulin secretion, inhibition of glucagon secretion as well as decelerating gastric emptying (Nadkarni et al., 2014). In addition, GLP-1 has other beneficial effects including promoting insulin gene transcription, stimulating pancreatic β-cell proliferation and neogenesis, inhibiting β-cell apoptosis (Manandhar et al., 2014). Variants like rs7901695 exerts its effect by deregulation of these GLP-1 and GLP-2 peptide expression in plasma (Doria et al., 2008).

HbA1C is glycosylation is a well-known hematological marker in poorly maintained T2D patients. Still this can be increased in normoglycemic patients with rs7901695 variants. Our analysis of HbA1C in diabetic patients over normal individual shows significantly high. Though this HbA1C level was not checked particularly within rs7901695 carrying patients but we presume that their individual level of HbA1C attributed to significance of HbA1C. Positively diagnosed diabetic patients are expected to be well maintained with their glucose level which may not show any significance is analysis. However, we presume that in our study population presence of large number of rs7901695 variants actually exerted the significance in HbA1C.

With people's changing life style and dietary habits, the prevalence of T2D mellitus has become much more serious than ever before. Among all the T2D related genes, the transcription factor 7 like 2 (*TCF7L2*) gene is one of the most relevant risk-related gene. Interesting fact is, unlike many other polymorphisms *TCF7L2* variants are manageable. Increased dietary fibre intake modifies the association between *TCF7L2* polymorphism and incidence of T2D (Hindy et al., 2012). Until now, several ethnic groups have been found to be associated T2D and this gene. Our case control study of rs7901695 has also found relevance with T2D in the Northern region of Bangladeshi population. This finding will help us for early diagnosis of the disease and early diagnosis will ultimately lead to better management.


Chapter 5
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Appendix

Certificate of Ethical Clearance

UNIVERSITY OF RAJSHAHI
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Rajshahi 6205, Bangladesh
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Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources

(Approved in the Resolution No. of the 71th meeting of the Board of Governors of the Institute of Biological Sciences and Resolution No. 57 of the meeting of the Syndicate of the University of Rajshahi)

Memo No: 59/320/IAMEBBC/IBSC 18 November, 2015

Certificate

This is to certify that the project title **"IDENTIFICATION OF TCF7L2 GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES"** Submitted by Dipa Roy, M.Phil Fellow, Institute of Biological Sciences, University of Rajshahi has been approved by the IAMEBBC in its resolution no. 06 of the 10th meeting held on 18th November, 2013.

Name of Chairman: Prof. Dr. M. Monzur Hossain

Monzur Hossain
24/11/15

Signature With Date

Consent Letter

for

Blood Sample Collection of Diabetic and Healthy Individuals

Institute of Biological Sciences, University of Rajshahi
Bangladesh

RESEARCH TITLE: IDENTIFICATION OF *TCF7L2* GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES IN NORTHERN REGION OF BANGLADESH

*I have heard about the research work from **Dipa Roy** and I decided to provide all Information and Blood without any terms and conditions.*

....
Sign of Donor and Date

সম্মতিপত্র

আমি নিম্নস্বাক্ষরকারি দীপা রায় এর নিকট তার গবেষণা বিষয় সম্পর্কে বিস্তারিত শুনিলাম এবং উক্ত গবেষণার স্বার্থে কোন প্রকার শর্ত ছাড়াই প্রয়োজনীয় তথ্যাদি এবং রক্তের নমুনা দিতে সম্মতি দান করিলাম।

....
স্বাক্ষর ও তারিখ

Data Collection Sheet for Research Purpose

Institute of Biological Sciences
University of Rajshahi
Rajshahi-6205, Bangladesh

RESEARCH TITLE: IDENTIFICATION OF *TCF7L2* GENETIC VARIANTS ASSOCIATED WITH TYPE 2 DIABETES IN NORTHERN REGION OF BANGLADESH

Sample No.	Age	Duration of symptoms	Other complications	FPG	BMI	Tg	Cholesterol	HbA1c