Master Program in Clinical laboratory Science

Molecular Characterization of Type 2 Diabetes Mellitus by Single Nucleotide Polymorphism of Transcription Factor7Like 2 Gene

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2013

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This thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Clinical Laboratory Science from the Faculty of Graduate Studies at Birzeit University Palestine

July 2013

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I dedicate this work to the memory of my beloved grandparents, my parents Khalil and Ruwaida, my husband Mousa, my sisters and brothers.

Acknowledgments

I would like to express my sincere gratitude to Dr. Tamer Essawi, the founder of the Master Program in Clinical laboratory Science for his motivation, enthusiasm, support and encouragement. His continuous guidance helped me throughout the course of this research project. I am deeply indebted to my supervisor, Dr. Mohammad Farraj who gave me the power to complete this thesis, for his immense knowledge who showed me how to work independently and prepared me for future challenges.

I would like to thank Mr. Israr Sabri for his help and Dr. Lina Al-Khairy for her valuable help in the statistical analysis of the data. I would also like to acknowledge Dr. Asaad Ramlawi, and Dr. Umaya Khammash who were extremely cooperative and provided me with the help to collect the samples I needed for my project. I would like to thank all my friends who supported me during this work.

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List of Abbreviations

T2DM	Type 2 diabetes mellitus
TCF7L2	Transcription factor-7–like 2
SNP	Single nucleotide polymorphism
WHO	World Health Organization
PPG	Postprandial plasma glucose
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IDDM	Insulin-dependent diabetes mellitus
NIDDM	Non-insulin-dependent diabetes mellitus
GDM	Gestational diabetes mellitus
GAD65	Glutamic acid decarboxylase
IDF	International diabetes Federation
NHANES	National Center for Health Statistics
OGTT	Oral glucose tolerance test
ESRD	End-stage renal disease
DPN	Diabetic polyneuropathy
DAN	Diabetic autonomic neuropathies
AGE	Advanced glycation end product
CAD	Coronary artery disease
PAD	Peripheral arterial disease
Wnt	Wingless-type integration site family
GLP	Glucagon-like-peptides

PCR	Polymerase chain reaction
RFLP-PCR	Restriction fragment length polymorphism – PCR
ASPCR	Allele-specific polymerase chain reaction
BMI	Body mass index
FBS	Fasting blood sugar
GIP	Glucose-dependent insulinotropic polypeptide
DPP-4	Dipeptidyl peptidase IV
OR	Odd ratio

Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is a multifactorial disease with a strong genetic component interacting with environmental factors. Many genes have been significantly associated with developing type 2 diabetes mellitus. Most of these genes have been linked to beta-cell dysfunction, impaired glucose homeostasis and insulin secretion. Transcription factor-7–like 2 (TCF7L2) gene has been found as an unexpected suspect for type 2 diabetes. The strongest and most commonly associated alleles of the TCF7L2 gene in T2DM in many countries are rs7903146 and rs12255372.

Aim: The aim of this study was to evaluate the association between TCF7L2 gene in T2DM among Palestinian people. Two SNP's rs7903146C/T, rs12255372G/T alleles in the TCF7L2 gene were investigated in diabetic patients and control groups.

Methods: This is a case control study. A total of 326 participants were included in this study; 249 participants with T2DM and 77 normal glycemic controls. RFLP PCR was performed using two restriction enzymes *Rsa*I and *Bse*GI to identify the presence of the two specific mutations in the alleles of the TCF7L2 gene among the study population. Allele specific PCR was also performed to substitute for DNA sequencing on one hand and to genotype the TCF7L2 gene as homo or heterozygous. We used SPSS v.21 to compare the results obtained of the case and control groups.

Results: There was a strong association between the two SNP's rs7903146C/T, rs12255372G/T alleles and T2DM. Both alleles have statistically significant association with the disease. Each of the two alleles had stronger association with T2DM when tested alone than when both alleles were combined. We observed that several normal participants carried the SNP in one or both alleles. This indicates the possibility of future development of diabetes.

Conclusion: TCF7L2gene is strongly associated with T2DM. This important finding can be utilized in screening the population at risk of developing diabetes. Furthermore, genetic testing can also be applied to the normal population to determine who can be prone to develop diabetes in the future.

الخلاصة

المقدمة: النوع الثاني من داء السكري (T2DM) هو مرض متعدد العوامل مع عنصر وراثي قوي التفاعل مع العوامل البيئية. وهناك العديد من الجينات المرتبطة بشكل كبير مع تطور النوع الثاني من داء السكري. وقد تم ربط معظم هذه الجينات إلى ضعف خلايا بيتا، وضعف توازن الجلوكوز وإفراز الأنسولين. حيث تم العثور على الجين (TCF7L2) باعتباره المشتبه به غير المتوقع لمرض السكري من النوع الثاني. وان الجينات الأقوى والأكثر شيوعا منها هي المرتبطة بالأليلات في الجين TCF7L2في T2DM في العديد من البلدان هي rs7903146 وrs12255372.

الهدف: لقد كان الهدف من هذه الدراسة هو تقييم العلاقة بين TCF7L2في T2DM بين الشعب الفلسطيني. وقد تم التحقق من اثنين منSNPفي الجين TCF7L2منهاrs7903146C/T، rs12255372G / Tفي مرضى السكري وغير المرضى (الطبيعيين).

الطريقة: هذه دراسة من نوع case control study. تم تضمين ما مجموعه ٣٢٦ مشاركا في هذه الدراسة؛ ٢٤٩ مشاركا من المرضى T2DMو ٧٧ من غير المرضى (نسبة سكر لديهم طبيعية في الدم). تم إجراء RFLP- PCR باستخدام اثنين من الانزيمات Rsa1 وBseG1 لتحديد وجود هذه الطفرات في الآليات من الجينات TCF7L2 بين المشتركين في الدراسة. تم إجراء PCRمحددة (Allele Specific PCR) أيضا على ان تكون بديلا عن تسلسل الحمض النووي من جهة، وإلى التركيب الوراثي للجين TCF7L2 كمتجانس أو غير متجانس. لقد استعمل برنامج الاحصاء (SPSS V.21) لمقارنة النتائج التي تم الحصول عليها بين الحالة قيد الدراسة ومجموعات المراقبة.

النتائج: كان هناك علاقة قوية بين الطفرتين rs7903146C/T، rs12255372G / T وتطور((T2DM). كلا الأليلات لديها ارتباط ذي دلالة إحصائية مع المرض. وأظهر كل من الأليلات الاثنتين رابطة أقوى مع النوع الثاني من السكري (T2DM) عند اختباره وحده مما كانت عليه عندما تم الجمع بين كل من الأليلات. وقد لاحظنا أن العديد من المشاركين غير المرضى حملواSNP في واحد اوفي كلا الأليلات التي اختبرت. وهذا يدل على إمكانية التطور المستقبلي لمرض السكري من قبلهم.

الخلاصة: الجين TCF7L2 يرتبط ارتباطا قويا مع النوع الثاني من السكري(T2DM) ويمكن استخدام هذا الاكتشاف المهم في فحص السكان المعرضين للخطر للإصابة بمرض السكري. وعلاوة على ذلك، يمكن أيضا أن تطبق الاختبارات الجينية للسكان العاديين لتحديد من الذين منهم عرضة لتطوير مرض السكري في المستقبل.

Chapter 1

Introduction

1.1 Early history of diabetes:

Diabetes mellitus is the most commonly encountered endocrine disorder worldwide (1). The history of diabetes has its beginnings in antiquity (Asia Minor, China, Egypt, and India). The ancient Egyptian civilization described diabetes as the passing of large quantities of honey-sweet urine, and was documented in the Ebers Papyrus (dating back to 1500 BC and discovered by the Egyptologist Georg Ebers in Thebes in 1872 (2, 3).

In the 2nd century AD, Aretaeus of Cappadocia used the term "diabetes" from the Greek word for "siphon" or "pass through" (4) .In the tenth century AD, Ibn Sina described the disease in his book Al-Qanoon and mentioned gangrene and collapse of sexual function as complications of the disease. In seventeenth century in England, Thomas Willis (1621-1675), indicated that the sweet taste of urine could be used by physician for the diagnosis of the disease. In 1766 Mathew Dobson proved that the sweet taste of urine in diabetics was due to sugar (5).

Paul Langerhans in 1869 discovered the islets of cells in pancreatic tissue and were later given his name "islet of Langerhans". Von Mering (1849-1908) and Oscar Minkowski (1858-1931)

hyperglycemia with ketoacidosis (mainly presents in type 1 diabetes) or the nonketotic hyperosmolar syndrome (it occurs more often in people with type 2, blood sugar levels rise, blood becomes thick and the body tries to get rid of the excess sugar by passing it into the urine) (9).

Classification of diabetes:

The first widely accepted classification of diabetes mellitus was published by WHO in 1980 (10) and then modified in 1985 (11). In 1980 there were two major classes of diabetes mellitus, insulin-dependent diabetes mellitus (IDDM or Type 1), and non-insulin-dependent diabetes mellitus (NIDDM or Type 2). In 1985, the terms Type 1 and Type 2 were omitted, but the classes IDDM and NIDDM were retained. In both the 1980 and 1985 reports other classes of diabetes were included such as Impaired Glucose Tolerance (IGT) and Gestational Diabetes Mellitus (GDM). These were reflected in the subsequent International Nomenclature of Diseases (IND) in 1991, and the tenth revision of the International Classification of Diseases (ICD-10) in 1992. The 1985 classification was widely accepted and used internationally. It represented a compromise between clinical and etiological classification, which allowed clinically useful classification of individual subjects and patients when the specific etiology was unknown. The recommended classification included staging of diabetes mellitus based on clinical descriptive criteria and a complementary etiological classification. The revised classification of WHO encompasses both clinical stages and etiological types of diabetes mellitus and other categories of hyperglycemia.

The clinical staging reflects that diabetes, regardless of its etiology, progresses through several clinical stages during its natural course. Moreover, individual subjects may move from stage to stage in either direction. People who have, or who are developing, diabetes mellitus can be categorized by a stage according to the clinical characteristics, even in the absence of information concerning the underlying etiology. The classification by etiological type results from improved understanding of the causes of diabetes mellitus (12).

The classification of diabetes includes both clinical and etiological causes are divided into four classes :(13)

- ***A*** **Type1diabetes** (IDDM), the disease tends to occur in childhood, adolescence or early adulthood (before age 30) but it may have its clinical onset at any age. It accounts for only 5–10% of the cases; it results from cellular-mediated autoimmune destruction of the-cells of the pancreas (usually leading to absolute insulin deficiency). Markers of the immune destruction of the-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatasesIA-2 and IA-2. In addition, the disease has strong HLA associations, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes, when the etiology of some forms of Type1 diabetes is not recognized, it is referred to as idiopathic diabetes (14).

- ***B*** **Type 2 diabetes** (NIDDM, type II or adult-onset) is the most common form of DM, it accounts for 90–95% of cases, it is characterized by hyperglycemia, insulin resistance, and relative insulin deficiency rather than absolute insulin deficiency. In Type 2 diabetes, the pancreatic β cells become progressively less able to secrete sufficient insulin to maintain normal carbohydrate and lipid homeostasis (15).It is highly associated with family history of diabetes, older age, obesity and lack of exercise (16). The etiology of type 2 diabetes mellitus is multifactorial; it may result from interaction between genetic, environmental and behavioral risk factors (17).

- ***C*** **Other specific types of diabetes**: (<5% of all diagnosed cases): due to other causes such as:
    - Genetic defect in beta cells.
    - Insulin resistance genetically determined.
    - Diseases of the exocrine pancreas,(Such as cystic fibrosis)
    - Hormonal defects.
    - Chemicals or drugs (such as in the treatment of HIV/AIDS or after organ transplantation) (18).
- ***D*** **Gestational diabetes mellitus** (GDM), this type is diagnosed during pregnancy mainly between 24 and 28 weeks of pregnancy, it occurs in 18 %of pregnancies. Women with a history of GDM have a high risk to develop diabetes, should be periodically maintained and tested (19).

1.3. Type 2 diabetes (non-insulin dependent DM- NIDDM, type II or adult-onset)

Type 2 diabetes will certainly be one of the major diseases of the 21st century (20).It was first described as a component of the metabolic syndrome in 1988 and the most common form of diabetes mellitus, which accounts for 90% of diabetic cases(21). Since Type 2 diabetes mellitus usually develops after the age of 40(although it is increasingly found in younger people), the disease is called "adult onset type diabetes mellitus".

NIDDM is characterized by hyperglycemia due to multiple defects in both insulin action and insulin secretion. Although many type 2 diabetes mellitus patients have a basal hyperinsulinaemia, rises in plasma glucose have a characteristically reduced stimulatory effect on insulin secretion. Insulin resistant the cells are unable to bind and internalize glucose. Insulin resistance indicates that cells are either malnourished or starved as sugar levels elevated in the blood. In response, the pancreas compensates for this by secreting more insulin in order to lower glucose levels. With time, beta cells may become completely damaged resulting in insulin insufficiency, eventually no insulin is produced, and a person with type 2 diabetes needs insulin injections for survival (22). Type 2 diabetes mellitus patients are often treated by diet or with oral hypoglycemic drugs, but many will eventually need exogenous insulin to reduce the levels of hyperglycemia (23).Initially, the disease is asymptomatic resulting in a large percentage of the patients not seeking early medical attention, thus 30-85% of cases of type 2 diabetes remain undiagnosed. At the time of eventual diagnosis, approximately 20% of patients will already have complications of the disease (24).

1.4 Discovery of Type 2 Diabetes:

The remarkable success of using insulin to treat diabetes led to the notion that the disease was caused by lack of insulin. Observations by Roger Hinsworth in 1935 resulted in the recognition of two different types of diabetes. Himsworth experiments in both animals and people concluded that the body's use of sugar depends on the availability of and the sensitivity of the cells to insulin. By giving diabetic patients sugar and insulin simultaneously, Himsworth determined the actual insulin effect on the sugar.

These experiments showed that there are two types of diabetes: Type 1 and Type 2. People with type 1 diabetes were sensitive to insulin and had a history of suddenly developing the disease at young age; those with type 2 diabetes were relatively insensitive to insulin and tended to gradually develop a milder form of the disease at middle age or older. Years later, other researchers confirmed Himsworth's findings with more sophisticated techniques. In the 50s, the work of a nuclear physicist and an internal medicine physician on diabetes research and treatment earned them the Nobel Prize. In the 1950s, Himsworth's notion that type 2 diabetes involved reduced sensitivity to insulin was not yet well accepted by the biomedical community, another researcher, Arthur Mirsky, explanation for adult-onset diabetes to be due to rapid enzymatic digestion of insulin. Rosalyn Yalow and Solomon Berson, researchers at New York's Veterans Administration Hospital, set out to test this hypothesis. They gave radioactively labeled insulin to people with and without diabetes. According to Mirsky's theory, the insulin given to those with diabetes should have disappeared more quickly than that given to normal individuals. Yalow and Berson found that it disappeared more slowly.

The slower rate of disappearance was due to an immune response against the radiolabeled insulin used in the experiments.

Radioimmunoassay was used to measure minute quantities of hormones (Pico / ml) of blood and other compounds circulating through the bloodstream. In 1960s, Yalow and Berson used the technique to measure and compare the insulin response to sugar in patients with type 2 diabetes and normal controls. They found that people with type 2 diabetes often generated more insulin than the normal controls. Other researchers discovered that although people in the early stages of type 2 diabetes produce more than the normal amounts of insulin, over time their insulin levels declines below that seen in normal individuals and their diabetes becomes severe. The outcome of these findings was a new hypothesis indicating that to compensate for their lack of sensitivity to insulin (insulin resistance), people with type 2diabetes initially produces excess insulin. That excess allows converting the sugar in the diet to energy needed by tissues. Eventually the insulin producing cells in the pancreas deteriorate and lose their ability to secrete insulin this indicates that external insulin must be used to treat the disease(25).

1.5 Prevalence of Type 2 Diabetes

T2D has become an epidemic in the 21st century. The total number of people worldwide with type 2 diabetes was expected to increase from 171 million in 2000 (2.8% of the world's population) to 366 million (4.4% of the world's population) in 2030 (26). Unfortunately, the prevalence worldwide has already reached 366 million by 2011 according to the International Diabetes Federation (IDF), 80% of these individuals live in low- and middle-income countries (26).The majority of the expected increase in T2D cases will occur in people aged 45 to 64 years living in developing countries, and 183 million people (50%) with diabetes are undiagnosed.

Clearly, the number of people with T2D diabetes is increasing in every country and the prevalence will continue to increase globally. It is projected that by 2030 the number would increase to 552 million .The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing obesity and physical inactivity (27).The increase in diabetes prevalence will have a negative impact on costs, type2 diabetes caused at least 465 billion dollars in healthcare expenditures in 2011; 11% of the total healthcare expenditures in adults (20-79 y) .The 'top' three countries in terms of the number of T2D cases are: China (90 million in 2000;129.7 million in 2030) India (61,3million in 2011;101 million in 2030), and the US (23.7 million in 2011; 29 million in 2030), and in Arab countries, the range between 4–21%, with the lowest being in Somalia and the highest in Kuwait (26).Six Arab countries are among the world's leaders in terms of type II diabetes prevalence: these countries are Kuwait, Lebanon, Qatar, Saudi Arabia, Bahrain, and United Arab Emirates (UAE) (24,28),as shown in Table (1).

In Palestine in 2004, the reported prevalence in Palestinian Family Health Survey was 10.6% (8.7–12.5) versus an estimated 11.4% (9.7–13.4); in 2006, these values were 11.8% (9.8–13.8) and 12.3% (10.6–14.6), respectively and by 2010, the prevalence of type 2 diabetes had

increased to 14.5% (12.2–16.7), in this period, prevalence in men rose from 11.7% (9.7–13.6) to 15.9% (13.4–18.1) and in women from 11.4% (9.3–13.3) to 13.2% (11.1–15.2). The forecasts for prevalence of diabetes are 20.8% (18.0–23.2) for 2020 and 23.4% (20.7–25.8) for 2030 (29).

Country	Population (×1,000)	Comparative* diabetes prevalence (%)	Male (×1000)	Female (×1000)
Kuwait	**1,868**	**21.2**	**175.3**	**122.6**
Lebanon	**2,788**	**20.1**	**230.9**	**296.9**
Qatar	**1,541**	**20.1**	**166.2**	**50.6**
Saudi Arabia	**17,023**	**20.0**	**1,450.7**	**1,308.8**
Bahrain	**986**	**19.8**	**91.4**	**59.3**
UAE	**6,107**	**19.2**	**296.2**	**128.9**
Egypt	**48,305**	**16.9**	**3,123.7**	**4,199.5**
Mexico	69,323	15.8	5,457.1	4,836.6
Libya	**3,875**	**14.1**	**211.2**	**225.1**
Jordan	**3,268**	**12.3**	**148.8**	**142.7**
Oman	**1,810**	**10.7**	**88.1**	**50.3**
Syria	**10,824**	**10.1**	**437.1**	**452.3**
Russia	109,166	10.0	5,227.4	7,365.6
Cypress	809	9.1	56.5	25.3
Yemen	**10,902**	**9.8**	**366.1**	**361.1**
Tunisia	**7,084**	**9.6**	**278.3**	**351.2**
USA	216,804	9.5	11,986.2	11,735.5
Iraq	**15,068**	**9.3**	**459.1**	**629.9**
OPT*	**1,896**	**9.3**	**51.8**	**72.6**
China	968,974	9.0	50,293.2	39,751.8
Sudan	**22,000**	**8.7**	**947.9**	**718.7**
Canada	25,140	8.6	1,479.7	1,236.4
India	737,003	8.3	32,498.1	28,760.3
Turkey	47,322	8.1	1,469.7	2,033.1
Italy	45,637	7.8	1,734.8	1,825.5
Israel	4,707	7.6	206.2	194.1
Algeria	**22,619**	**7.0**	**704.4**	**730.7**
Morocco	**19,964**	**6.9**	**608.7**	**659.1**
Djibouti	**480.9**	**6.4**	**13.1**	**13.1**
Germany	62,810	5.5	2,674.2	2,347.9
France	44,328	5.5	1,733.8	1,503.7
UK	44,813	5.3	1,790.1	1,273.8
Mauritania	**1,756**	**4.3**	**29.1**	**31.4**
Somalia	**4,275**	**4.2**	**87.6**	**97.4**

* All comparisons should be done using the comparative prevalence, which is adjusted to the world population.

Table 1.Ranking of the prevalence of type 2diabetes in Arabic and non-Arabic speaking countries International Diabetes Federation using IDF estimates for 2011, Arabic speaking countries are shown in bold. IDF Diabetes Atlas (26).

1.6 Etiology of Type 2 Diabetes and Risk Factors

There are many different causes of this form of diabetes. Although the specific etiologies are unknown, type 2 diabetes can be caused by environmental (include behavioral factors) and genetic factors, insulin sensitivity and insulin secretion (30, 31).Although genes that predispose an individual to diabetes are considered to be an essential factor in the development of the disease, activation of a genetic predisposition is usually triggered by environmental factors, particularly those who are overweight with low physical inactivity and gestational diabetes (32).

The risk factors associated with type II diabetes can be grouped into two categories: modifiable and non-modifiable risk factors (33).

a. Modifiable Risk Factors:

Overweight and obesity (defined by a body mass index) has a strong relationship with type 2 diabetes and it is the main risk factor for NIDDM because it plays essential role in causing insulin resistance (34). According to National Center for Health Statistics (NHANES) 78.5% of diabetics are overweight, and 45.7% are obese (35).

Sedentary lifestyle: many epidemiologic studies show that increasing physical activity reduces risk of diabetes, whereas sedentary behaviors increase the risk such as prolonged television (TV) watching (36) extended time sitting, even with regular periods of exercise, they are still at greater risk for diabetes (37).

Previously identified glucose intolerance (IGT and/or IFG): impaired fasting glucose (IFG, fasting plasma glucose > 6.1 and < 7.0 mmol/L) here the blood glucose levels that are higher

than normal, but below the level of a person with diabetes also Impaired Glucose Tolerance(IGT; two-hour plasma glucose > 7.8 and < 11.1 mmol/L) (38).About 40-50% of people with IGT will develop type 2 diabetes (accompanied by increased risk of cardiovascular disease and microvascular complications) within ten years (39).

b. Non-modifiable risk factors:

Ethnicity: The risk of Type 2 diabetes is greater in African-American, Hispanic/Latin American, American Indian and Alaska Native, Asian-American, or Pacific Islander ethnicity. In some studies, the researchers found that the prevalence of diabetes was 16.1% among Native Hawaiians, 15.8% in Latinos, 15% in African-Americans, 10.2% in Japanese-Americans, and 6.3% in whites. The reasons why people of these specific ethnicities are at greater risk of type 2 diabetes are not fully understood and may be attributed to genetic effect (40).

Family history of Type 2 diabetes: A family history of diabetes has a stronger risk factor for Type 2 diabetes than type 1. Having one or more first-degree relatives with T2DM increases the odds of having the disease compared with someone without such relatives. The estimations vary, but the odds usually range from two to six times more likely (41).Since genetic tests are not currently available for type 2 of diabetes; the use of tools that include a family history of diabetes is potentially applicable way (42).

Age: The chance of getting Type 2 diabetes increases with age. That's probably because people tend to exercise less, and gain weight as they age. There are combined effects of increasing insulin resistance and impaired pancreatic islet function with advanced age (43).However, there is age-related declines of pancreatic islet function and islet proliferative capacity (44).

History of gestational diabetes: GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (45).Screening for GDM is performed with a 50g oral glucose load given between 24 and 28 weeks gestation, followed by measuring the plasma glucose load after one hour. If the level is >140 mg/dl, the patient is scheduled for a 3-hours, 100g oral glucose tolerance test (OGTT).

The following are the values considered to be abnormal during the 100 g of glucose OGTT:

Fasting blood glucose level ≥95 mg/dl (5.33 mmol/L)

1 hour blood glucose level ≥180 mg/dl (10 mmol/L)

2 hour blood glucose level ≥155 mg/dl (8.6 mmol/L)

3 hour blood glucose level ≥140 mg/dl (7.8 mmol/L) (46)

It is a risk factor for Type 2 diabetes in the mother after pregnancy (47). About 30% of women will develop diabetes within 15 years. Therefore, regular screening for Type 2 diabetes is essential for women who have GDM (48), they have a 7.5-fold increased risk for the development of type 2 diabetes after delivery (49).

1.7 Complications of diabetes mellitus

Diabetes or hyperglycemia over many years leads to damage to several tissues in the body, producing so-called diabetic complications and they are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, retinopathy and dermopathy) as shown in Figure2(50).

Microvascular Complications of Diabetes

Diabetic retinopathy Blindness is one of the most common microvascular complications of diabetes resulting in blindness for over 10,000 people with diabetes per year. Twenty years after the onset of diabetes over 60% of patients with type 2 diabetes will have some degree of retinopathy. Even at the time of diagnosis of type 2 diabetes, about a quarter of patients have established background retinopathy, and there is evidence that retinopathy begins to develop at least 7 years before the clinical diagnosis of type 2 diabetes.

Diabetic retinopathy is due to microangiopathy affecting the retinal pre-capillary arterioles, capillaries, and venules. Damage is caused by both microvascular leakages from breakdown of the inner blood-retinal barrier and microvascular occlusion. (51, 52)

Diabetic nephropathy: Diabetes has become the most common single cause of end-stage renal disease (ESRD) in the U.S. and Europe. The earliest clinical evidence of nephropathy is the appearance of low but abnormal levels of albumin in the urine (≥ 30 mg/day or 20 μg/min), referred to as microalbuminuria, thus diabetic nephropathy has been categorized into stages

based on the values of urinary albumin excretion (UAE): microalbuminuria and macroalbuminuria (53).

A higher prevalence of type 2 diabetes is found to have microalbuminuria and then they will have nephropathy after the diagnosis of their diabetes. Because diabetes is actually present for many years before the diagnosis is made and because the presence of albuminuria may be less specific for the presence of diabetic nephropathy, 20–40% of type 2 diabetic patients with microalbuminuria progress to overt nephropathy, but by 20 years after onset of overt nephropathy, only ~20% will have progressed to ESRD (54).

Diabetic neuropathy: The diabetic neuropathies affect different parts of the nervous system and they may be focal or diffuse. Most common among the neuropathies are chronic sensorimotor distal symmetric polyneuropathy (DPN) and the autonomic neuropathies (DAN).

Many mechanisms which are related to hyperglycemia such as polyol accumulation, injury from AGEs (advanced glycationend product), and oxidative stress may cause the injury to the peripheral nerves, so the -risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycemia.

More than 80% of amputations occur after foot ulceration or injury, which can result from diabetic neuropathy. The considerable morbidity and mortality that can result from diabetic neuropathy, it is important for clinicians to understand its manifestations, prevention, and treatment (55).

Classification of diabetic neuropathy

Peripheral neuropathy

Distal symmetric polyneuropathy (DPN) is defined of DPN for clinical practice as “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” is present in at least 20% of adult diabetic patients.

Cranial neuropathy

Is extremely rare (0.05%), cranial nerves affected 111, 1V, V1,V11 , and are thought to occur due to a microvascular “infarct.

Autonomic neuropathy

This condition results in significant morbidity and may lead to mortality in some patients with diabetes. This neuropathy may lead to: postural hypotension, resting tachycardia, loss of sweating , gastrointestinal neuropathy such as gastroparesis, diabetic diarrhea or constipation , urinary bladder atony and erectile dysfunction: The prevalence of erectile dysfunction in diabetic men ranges from 27 to 75% (56).

Macrovascular Complications of Diabetes:

Type 2 diabetic patients are at a high risk for macrovascular complications, including diseases of coronary arteries, peripheral arteries, and carotid vessels.

The central pathological mechanism in macrovascular disease is atherosclerosis, which leads to narrowing of arterial walls throughout the body .In diabetes; hyperglycemia, excess free fatty acid release, render arteries susceptible to atherosclerosis, impairs endothelial function, vasoconstriction increases inflammation, and promotes thrombosis. In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries. Angiotensin II may promote the oxidation of such particles then activation of the transcription factors nuclear factor κB (NF-κB) and activator protein 1 induces inflammatory gene expression, as a result increased production of inflammatory cytokines, and augmented expression of cellular adhesion molecules. Monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes.

T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The net result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of this lesion usually leads to acute vascular infarction, as shown in Figure 1 (50).

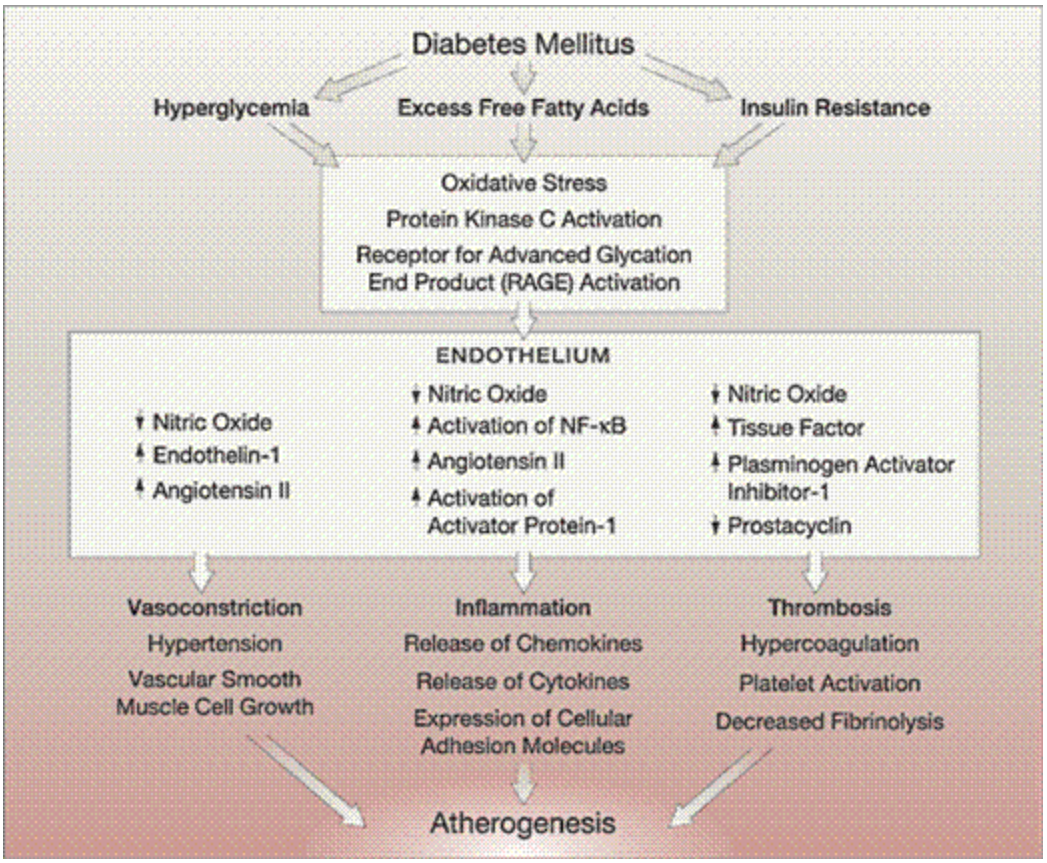

Figure 1. Endothelial Dysfunction in Diabetes. (The Journal of the American Medical Association JAMA. 2002 (57)).

Classifications of Atherosclerosis:

Coronary artery disease (CAD) causes much of the serious morbidity and mortality in patients with diabetes, who have a 2- to 4-fold increase in the risk of CAD. The amplified incidence of CAD results from the aggregation of multiple risk factors, such as obesity, dyslipidemia, and hypertension, which occur in this population (58).

Peripheral arterial disease (PAD) Individuals with diabetes have a 2- to 4-fold increase in the rates of PAD, more often have femoral bruits and absent pedal pulses, and have rates of abnormal ankle-brachial indices ranging from 11.9% to 16%.

Cerebrovascular Disease and its effects in the coronary and lower extremity vasculature: Patients with diabetes have more extracranial atherosclerosis .Diabetes affects stroke outcome as well so it increases the risk of stroke-related dementia more than 3-fold, doubles the risk of recurrence, and increases total and stroke-related mortality (59).

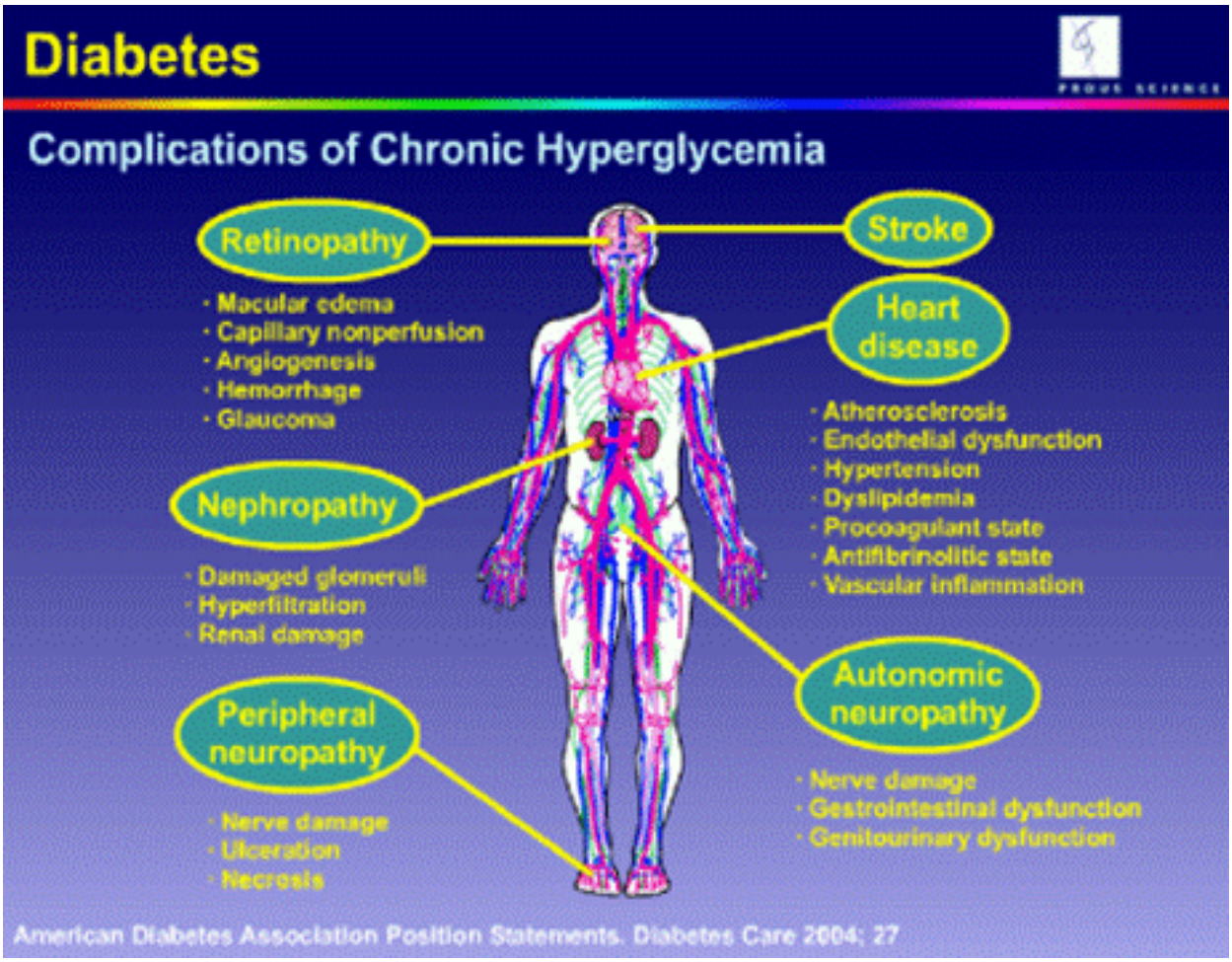

Figure (2): Diabetes complications. (Diabetes care 2004,27)(60).

1.8 Genes and Type 2 Diabetes

It has been known that T2D is a multifactorial disease with a strong genetic component interacting with environmental factors (61). Inheritance is a strong factor implicating inheritable genetics in type 2 diabetes: in monozygotic twins (96%) supports a substantial contribution of genetic factors to T2D (62) also 40% having relatives (especially first degree) with type 2 increases the risks substantially (63, 64). Many genes have been significantly associated with developing type 2 diabetes, until 2011 more than 36 genes have been found that contribute to the risk of type 2 diabetes, most of the discovered gene variants have been linked to beta-cell dysfunction, impaired glucose homeostasis and insulin secretion rather than insulin resistance (65).

From these genes there are18 genes that affect β-cell function, namely :CAPN10(66) ,CDC123/CAMK1D (67), CDKAL1 , CDKN2A/B(68) , ENPP1(69) ,FOXO1(70), HHEX(71), IGF2BP2, JAZF1 (72),KCNJ11(73) ,KCNQ1(74) ,MTNR1B(75), PPARGC1A(76) ,SGK1(77) , SLC30A8 (78), TCF7L2(79) , TSPAN8/LGR5 (80), and WFS1(81). The most promising candidate genes for human T2DM susceptibility arising from association studies are shown in Figure 3 on the next page:

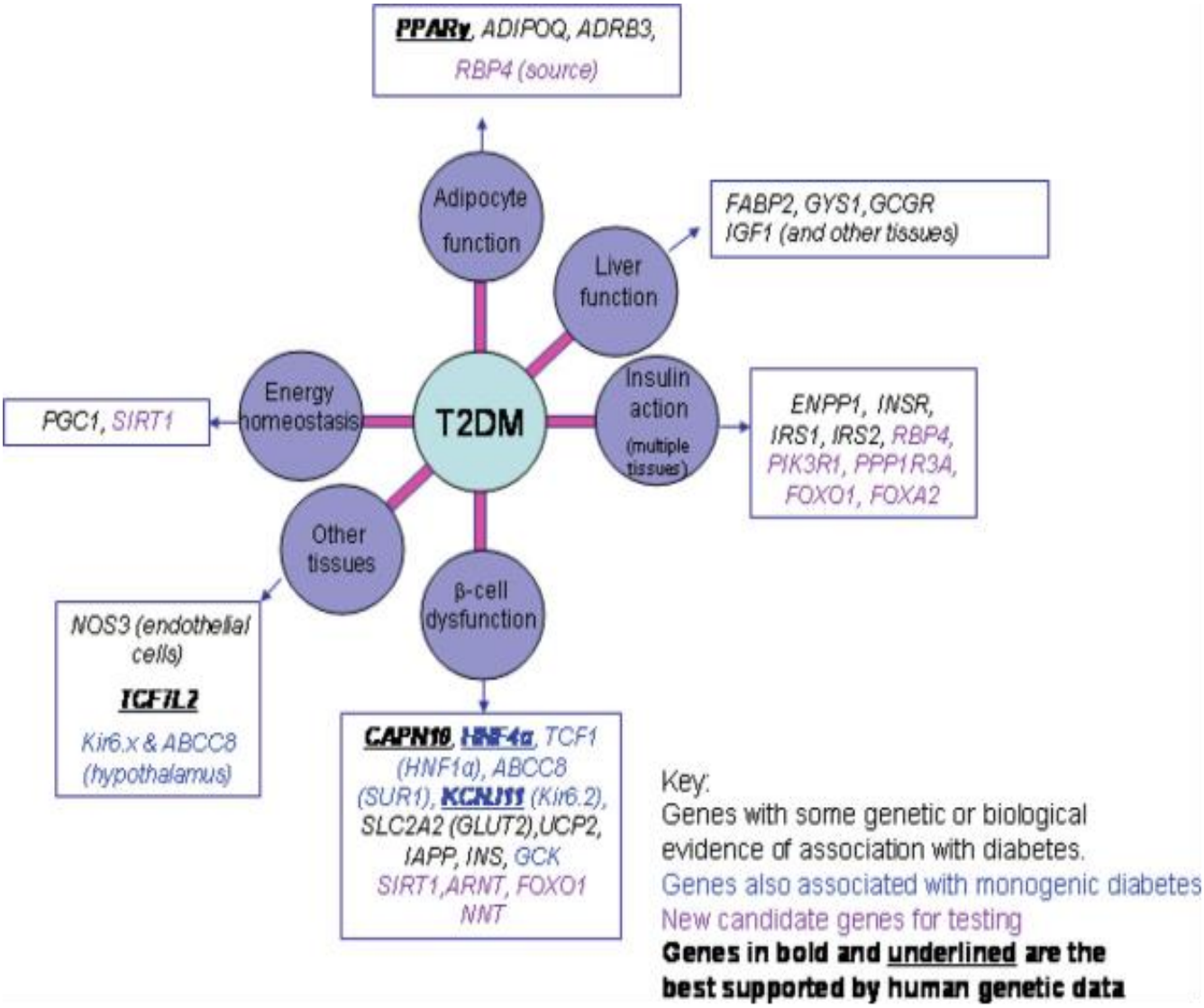

Figure 3: Biological function of T2DM candidate genes arising from animal models and human association studies. Many genes have been associated with T2DM (Human Molecular Genetics, 2006, Vol. 15)

1.9 *TCF7L2*, the most important type 2 diabetes gene:

Prior to 2006, only two genes were implicated in type 2 diabetes (PPARG1 and KCNJ11) (84). After 2006, transcription factor-7–like 2 gene (TCF7L2) has been found as an unexpected suspect for type 2 diabetes by the DECODE group in Iceland (85). They reported that a common microsatellite in TCF7L2 gene region DG10S478 within intron 3 was associated with type 2 diabetes in Icelandic case control sample. The overall estimated allelic relative risk was 1.56 with P value of 7.8×10^{-5} and the noncoding SNPs rs7903146 (C/T), and rs12255372 (G/T) were in strong linkage disequilibrium with DG10S478 (r^2=o.95 and 0.78 respectively) (86). They then genotyped three extra single nucleotide polymorphisms (SNPs) with the strong correlation to DG10S478 (rs7901695, rs11196205, and rs7895340) and showed association between all three SNPs and type 2 diabetes but the rs7903146 and rs12255372 were the strongest.(87,88) Subsequently, this association was confirmed in other populations such as Indians (89), French (90), Tunisians (91), Japanese (92), Mexican Americans (93), and West Africans (94)Amish (87), Polish (95) Chinese (96), Swedish(97) , Persian (98),and Palestine (99),all of these studies showed a strong association between TCF7L2 polymorphism and type 2 diabetes.

Several genome wide association studies independently confirmed the presence of strong association of SNP rs7903146 of TCF7L2 with T2D. These data demonstrated that the genetic variants within TCF7L2 gene, especially T allele of SNP rs7903146, are associated with the risk of developing T2D in several ethnic groups (100).Specific variations in TCF7L2 gene increase risk of type 2 diabetes by 1.5 folds in heterozygotes and 2.4 folds in homozygotes, corresponding to a population attributable risk of 21%. This makes TCF7L2 variants the strongest known genetic risk factor for type 2 diabetes (101).

1.10 The mechanism by which TCF7L2 alleles predispose to diabetes

TCF7L2 (also known as TCF-4)gene is a member of the T-cell factor (TCF)/lymphoid enhancing factor family of high mobility group box-containing transcription factors .It is a transcription factor and key component of the Wingless-type integration site family (Wnt) signaling pathway, which exerts many important physiological and pathophysiological functions in different cell lineages and organs (101) .Signaling is initiated by the binding of Wnts to their receptor complex, which results in the release of beta -catenin from its degradation complex and translocation to the nucleus. In the nucleus, beta -catenin heterodimerizes with the TCF/lymphoid-enhancing factor family of transcription factors to regulate the expression of Wnt target genes like proglucagon and glucagon-like peptides 1 and 2 (102, 103) ,as shown in figure (4) . Potential mechanisms by which TCF7L2 variants influence type 2 diabetes include its role in adipogenesis, myogenesis, and pancreatic islet development, as well as transcription of the genes for proglucagon and the glucagon-like-peptides GLP-1 and GLP-2, which play a role in post-prandial insulin secretion(104). Additionally, TCF7L2 polymorphisms have been associated with impaired insulin secretion via direct effects on pancreatic islet beta cells (105,106), because it is required for β-cell survival and β-cell proliferation (107) so the polymorphism in this gene will increase the risk of diabetes by affecting insulin secretion, not insulin resistance (108), as shown in Figures(5,6). Thus, while the specific mechanism driving the development of type 2 diabetes remains unclear, there is sufficient evidence to demonstrate that TCF7L2 variants strongly predict the development of type 2 diabetes and/or the progression to diabetes from impaired glucose tolerance (106,109)

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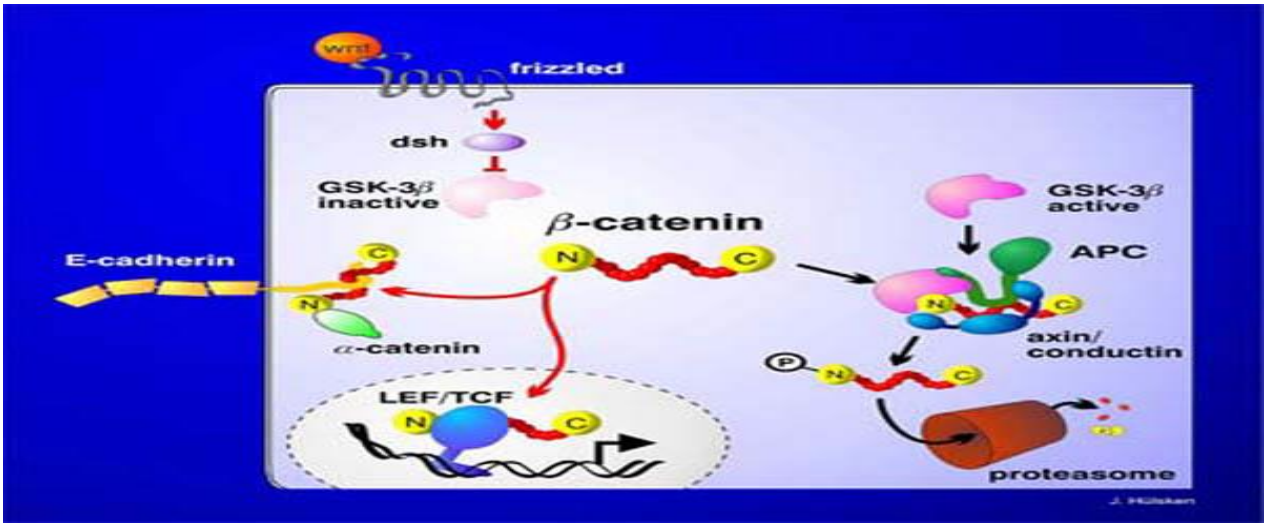


Figure (4): The Wnt Signaling Pathway. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to stabilization of hypophosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription. In a canonical pathway, CKIα, GSK3β, APC, and Axin act as negative regulators and all other components act positively (Max Delbruck Center for Molecular Medicine (MDC) Berlin-Buch 2007)

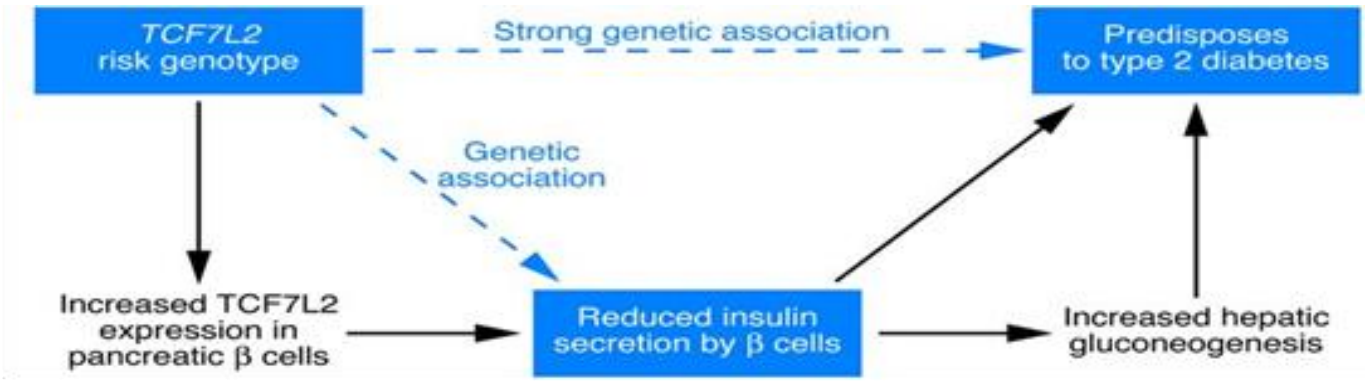


Figure (5): proposed pathophysiological pathway explaining how TCF7L2 risk genotypes predispose to type 2 diabetes. The risk genotype results in over expression of TCF7L2 in pancreatic β cells, which in turn results in reduced insulin secretion. Reduced insulin secretion results in a predisposition to type 2 diabetes directly and also indirectly by increasing hepatic glucose output (110).

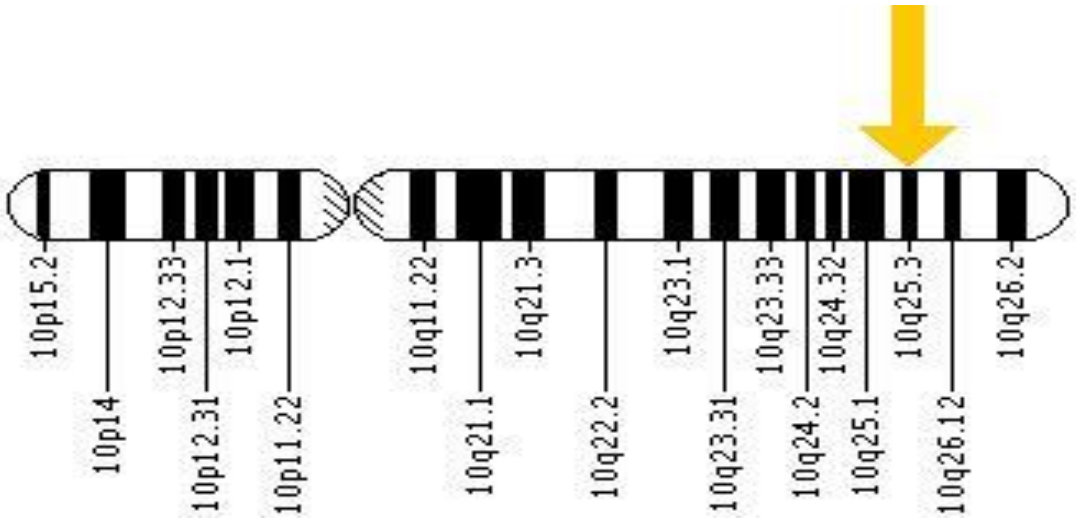


Figure (6): The TCF7L2 gene is located on the long (q) arm of chromosome 10 at position 25.3 (ghr.nlm.nih.gov) (111).

Aims of the study:

In this study we will focus on the Transcription Factor7Like2 gene (TCF7L2).It is a candidate gene which strongly related type 2 diabetes (T2D) locus identified to date (82). Common variants in this gene have a marked and reproducible effect on type 2 diabetes risk. This variation occurs at a single nucleotide polymorphism (SNP) within TCF7L2 (83).

The aims of this study are the following:

1. To determine the presence of association between T2DM and TCF7L2.
2. To determine the rate of SNP rs7903146, and rs12255372 in patients with T2DM and compare that with normal non-diabetic controls.
3. To determine the significance of the two SNPs individually and combined in T2DM patients as compared to controls.
4. To determine the possibility of conducting genetic testing on TCF7L2 gene in predicting possible development of diabetes in healthy people.

Chapter 2

Materials and Methods

2.1 Sample Collection

A total of 326 samples were collected from the West Bank and East Jerusalem. Blood was collected from all participants using vacutainer tubes with EDTA. Aseptic technique was followed to ensure patient safety and to minimize harm. All samples were stored at -80 ^{o}C until further DNA purification and subsequent molecular applications. The distribution of the samples is summarized in Table (2), Data collected for each sample included: age, gender, BMI, FBS, total cholesterol, blood pressure and family history of 1st degree relatives with T2D. This information is shown in Table (4) in the appendix.

Table (2): Distribution of samples on the different regions of the West Bank and Jerusalem

City	**Clinic**	**Specimens**
Jerusalem	UNRWA Clinic – Indian Hospice	60
Bethlehem – South West Bank	UNRWA Clinic- Khamashta	58
Ramallah – Central West Bank	Ministry of Health Clinics	46
	Diabetes Society – Al Bireh Center	18
North West Bank	Nablus – Medicare Labs	15
	Tulkarem – Ministry of Health Clinics	11
	Qalqelia – Ministry of Health Clinics	41
Controls	Clinics throughout West Bank and Jerusalem	77
TOTAL		**326**

2.2. DNA Extraction:

DNA was extracted from all samples by the salting out method (112,113). Briefly, white blood cells were lysed by lysing buffers; the supernatant was digested with Proteinase K at 56 ° C for 24 hours followed by salting out of the proteins by 5.3 M NaCl. The DNA fraction in the supernatant was purified by 100% ethanol for 24 hours at -20 ° C and treated with 70% ethanol, dried and dissolved in Tris-HCL. The quality and quantity of the extracted DNA was checked by electrophoresis on 1% agarose as shown in Figure (7), and the DNA was stored at -20 ° C for further work.

2.3. Polymerase Chain Reaction (PCR):

Amplification was conducted in a total volume of 25 µL. The2X ready PCR mix used for amplification (Thermo Scientific) consisted of: 1.25U Taq-Pol, 75 mMTris-HCL (pH 8.8), 1.5 mM $MgCl_2$, and 0.2mM of each dNTP. The reaction mixture contained: 12.5 ul master mix, 1.0 uM each forward and reverse primers (Table 3), 1ug DNA template and 8.5 ul RNase free water to a total volume of 25 ul. The amplification was carried out in a C-1000 thermal cycler (Biorad, USA) according to the following program: an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 59 ° C or at 64 ° C (depending on the primer used) for 1 min, and a final extension step at 72° C for 5 min. Amplified PCR products were resolved by agarose gel electrophoresis (5V/60 min)using 1,5% agarose in Tris Acetate-EDTA (TAE) buffer containing 0.5 ug/ml of ethidium bromide. Molecular size ladder of 100 bp (Fermentans, Germany) was used to determine the size of the bands. The gel was viewed and photographed on a Gel-Doc System (BioRad, USA),as show in

Figures (8,9).The primers used for the amplifications were obtained from Invitrogen (Rhenium, Jerusalem) and summarized in Table (3).

Primer	5' – 3' sequence	Product size (bp)	Annealing (°C)	Ref.
Forward Reverse For SNP rs12255372	5′-CCCAGGAATATCCAGGCAAGGAT-3′ 5′-CAAATGGAGGCTGAATCTGGCA-3′	120	59	114
Forward Reverse For SNP rs7903146	5′-TTAGAGAGCTAAGCACTTTTTAGGTA-3′ 5′ACTAAGTTACTTGCCTTCCCTG-3′	120	64	114

Table 3. PCR primers used for the amplification of single nucleotide polymorphisms of the *TCF7L2* gene (114).

2.4. Restriction Fragment Length Polymorphism – PCR (RFLP-PCR)

Restriction of the 120 base pairs PCR products was performed using the restriction enzymes *Rsa*I(Thermo) for the SNP - rs7903146, and *Bse*GI (Thermo) for the SNPrs12255372, following the manufacturer's instructions. Specifically, 7.5 ul of the PCR product was suspended in 1X diluted buffer (BSA-provided with the enzyme) and incubated with 1.5U of the enzyme for 2 hours at 37° for *RsaI* and 55°C for *BseGI* and 3% Agarose gel was used to separate the restriction fragments after digestion with the restriction enzyme. The C allele creates a restriction site and it gives two fragments less than 100 base pairs in non-mutated genes ,the results were viewed on Gel-Doc System (BioRad, USA) and photographed, as shown in Figure (10,11),and the results are summarized on Table (4) in the appendix.

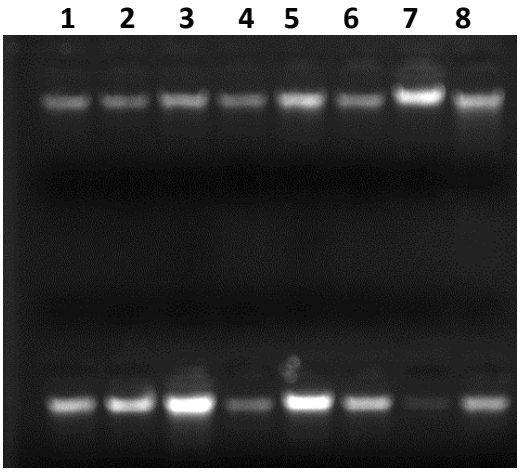


Figure 7:
Lanes 1 to 8 representing different samples with DNA extraction by this procedure

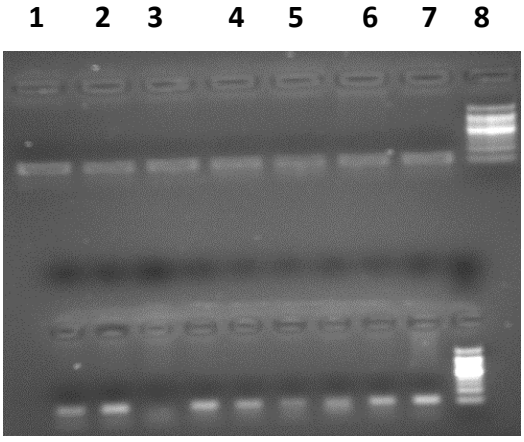


Figure 8: PCR product for **rs7903146,** Lanes 1 to 7 represents samples for PCR products for rs**7903146**, Lane 8, 100 bp ladder.

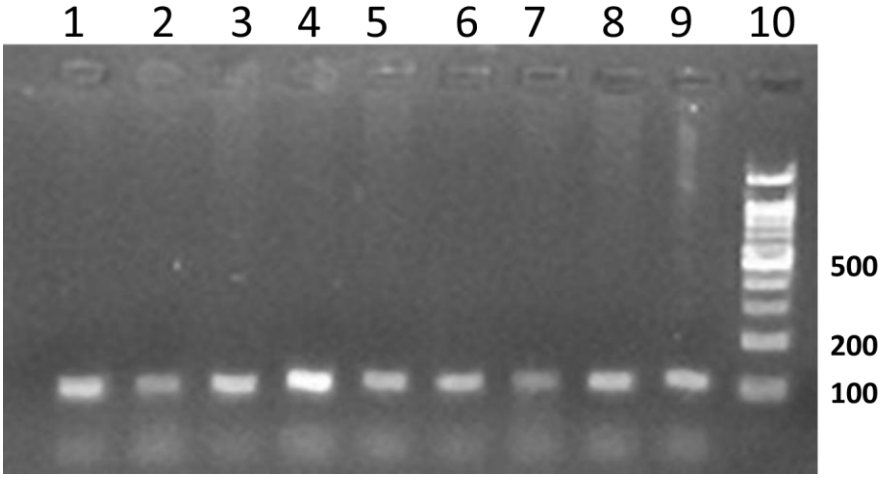


Figure 9: PCR product for **rs12255372.**

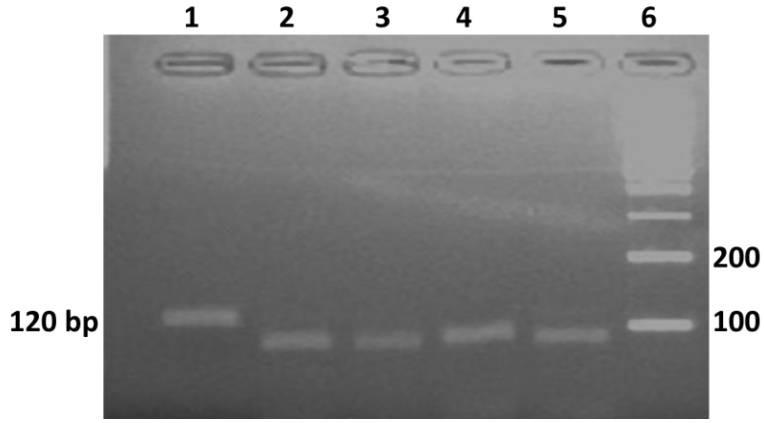


Figure 10: RFLP –PCR for rs7903146, the four control samples the band from right to left (non-mutated) less than 100 base pair (bp) and the fifth band for single patient more than 100bp (mutated)

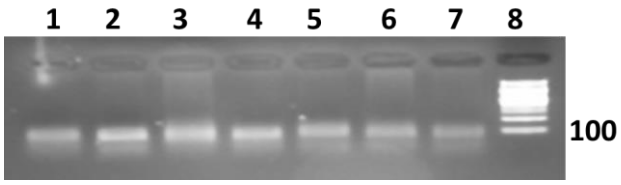


Figure 11: RFLP –PCR for rs12255372, Lanes 1 to 7 are Cases of mutated alleles with a band size of 120 bp.

2.5 Allele-specific polymerase chain reaction (ASPCR): The forward PCR primers were used for allele-specific PCR, for both mutations. The last nucleotide of the forward primer at 3'end was changed as shown in the ncbi sequence of the TCF7L2 gene below. The forward modified primer and the unchanged primer were tested with the reverse primer. This allowed us to confirm the presence of the mutations detected previously by the RFLP PCR.

FOR rs12255372

tggcttgcaggtcagattttcatctttttaaattaattatcatagaaggagaaaacaactggatttcagaattgtcccttgaggtgtactggaaact
aaggcgtgagggactcataggggtctggcttggaaagtgtattgctatgtccagtttacacataaggatgtgcaaatccagcaggttagctga
gctgcccaggaatatccaggcaagaatgaccatattctgataattactcaggcctctgcctcatctccgctgccccccgcccccctgactctct
tctgagtgccagattcagcctccatttgaatgccaaatagacaggaaattagcatgcccagaatccacgtctttagtgcactctctccccagct
ccaaacctgttactgcttgtgttcaacatctcagtaaagctcaacaacatcgacccattacttaggcctcaaaccttgggtggcatcgtcgattg
ctctttt.(115).

Primers:
Forward Normal:ccaggaatatccaggcaagaatg
ForwardMutated:ccaggaatatccaggcaagaatt

For RS rs7903146

TAATTGTAAATTGAATYGGACTAAAACCTTTCCAATTTTTTCAYATGTGAAGACATACACAAAA
GTTTTATTGGAGGGTTGCACATGTGAAAGAAAAAGGGAGAAAGCAGGATTGAGCAGGGGGAGCC
GTCAGATGGTAATGCAGATGTGATGAGATCTCTGCCRGACCAAAGAGAAGATTCCTTTTTAAAT
GGTGACAAATTCATGGGCTTTCTCTGCCTCAAAASCTAGCACAGCTGTTATTTACTGAACAATT
AGAGAGCTAAGCACTTTTTAGATACTATATAATTTAATTGCCGTATGAGGCACCCTTAGTTTTC
AGACGAGAAACCACAGTTACAGGGAAGGCAAGTAACTTAGTCAATGTCAGATAACTAGGAAAAG
GTTAGAGGRGCCCTGGACACAGGCCTGTRTGACTGAGAAGCTTGGGCACTTCACTGCTACATTT
CATYTCTTCGCTATAAACATTTTAGCTTTTTG(115).

Forward Normal:AGAGAGCTAAGCACTTTTTAGATAC
Forward Mutated:AGAGAGCTAAGCACTTTTTAGATAT

Note: the same PCR reverse primers for both mutations were reused.

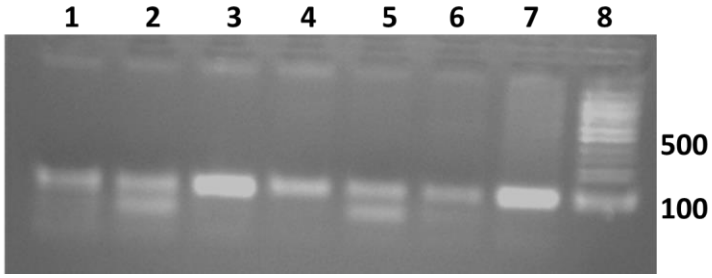


Figure 12: Allele specific PCR for rs7903146, Lanes
1 to 7 are heterozygous samples with normal and mutated bands.

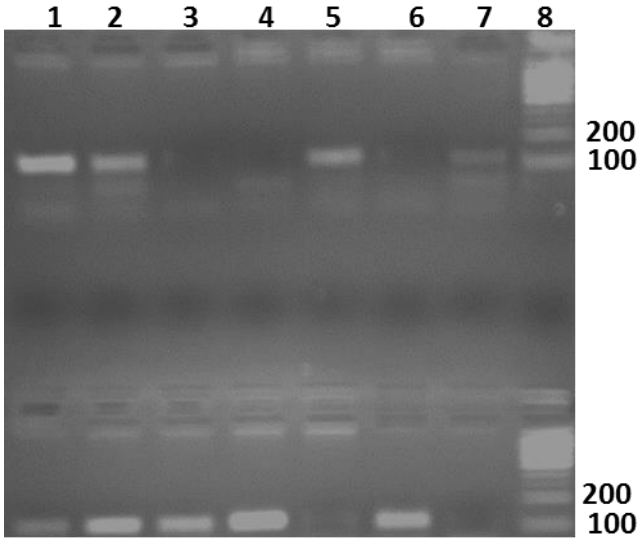


Figure 13: Allele specific PCR rs7903146, above row from right to the left:(1)Normal CC, (2,3),homo-mutated sample TT, (4,5)normal sample CC ,(6,7)sample with mutated and normal bands hetero-mutated CT.

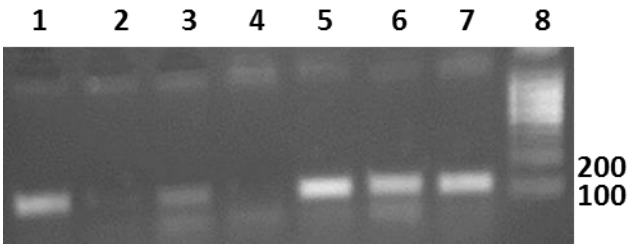


Figure 14: Allele specific PCR rs12255372

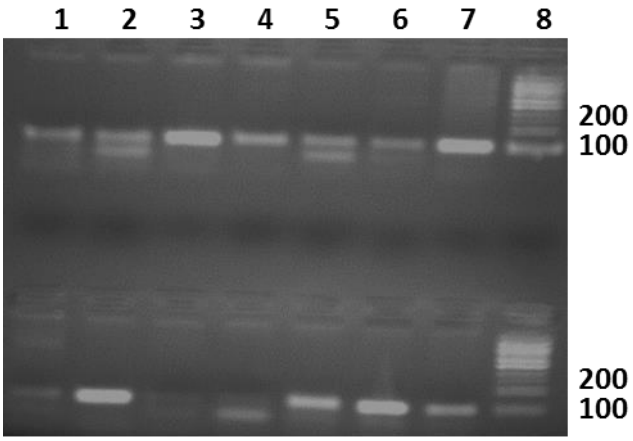


Figure 15: Allele specific PCR rs12255372
Above row 1-7 all are heterozygous (GT) samples.

2.6 Statistical analysis:

The data were analyzed using the SPSS v.21 program (Chicago, IL). The descriptive statistics were used to calculate frequencies: mean, standard deviation for all the variables. The independent-samples t-test was used to compare the differences between means. Some of the continuous variables were converted into categorical variables: **age** (30-50 years ,51-70years ,71-85 years, **diastolic** blood pressure (within 60-95mmHg , greater than 96-110mmHg), **systolic** blood pressure (within 90-139 mmHg , greater than140-210 mmHg), **Total cholesterol** within **(**40-239 mg/dL, greater than 240-400 mg/dL),body mass index **BMI (**18-24.9(kg/m^2),25- 29.9 (kg/m^2) ,30-55(kg/m^2)),and **fasting blood sugar** FBS(less than 125 mg/dL, greater than 126 mg/dL). Then the logistic regression was used to predict disease status (diabetes) and to adjust for different variables (covariates). *P -value* less than 0.05 was considered significant.

Chapter 3

Results

In this case-control study a total of 326 participants were analyzed first by Restriction Fragment Length Polymorphism – PCR (RFLP-PCR) for TCF7L2 gene to detect the presence of SNPs for both sites rs7903146 and rs12255372. There were 47.4% (118/249) of the cases tested were positive for the mutation **rs7903146 and** 52.6 % (131/249) were negative. In the **controls,** there were 15.6% (12/77) positive and 84.4% (65/77) negative. For **rs12255372**: in **cases**37.8% (94/249) were positive, 62.2% (155/249) were negative and in **controls** 9.1% (7/77) were positive, 90.9% (70/77) were negative.

By using the t-test ,mean age for cases and controls was significantly different ,mean age for the cases was 58.66 ±9.59 years and the controls was 42.99 ±8.12 years. In this study, there was no significant difference between cases and controls regarding the gender of the participants.

Mean BMI was 26.49 ± 3.54 kg/m^2 for controls and 31.34±5.73 for cases. There was significant difference detected between the cases and controls at 0.05 level of significance ($P < 0.005$). Systolic blood pressure , Family history of type 2 diabetes, fasting blood sugar FBS and total cholesterol all of them showed significant difference between cases and controls at 0.05 level of significance ($P < 0.005$) except for the diastolic blood pressure was not statistically significant . All of these data are summarized in Table **(5).**

By using RFLP-PCR, after adjusting for all risk factors and exclusion family history and FBS the logistic regression yielded significant odds ratio suggesting that the risk alleles confers significant risk for developing T2DM with reference to both SNPs: rs7903146 and rs12255372.

For rs7903146 odd ratio OR=3.0 (95%CI 1.24-7.24), rs12255372 OR= 5.5 (95%CI 1.97-15.19), before adjusting for all risk factors the results also were significant for rs7903146 OR= 4.88 (95%CI 2.51-9.48) and rs12255372 OR=6.065 (95%CI 2.68-13.74). When the family history was included in the statistical analysis, the OR was 2.1(95%CI 0.81-5.2) for rs7903146 and 3.3(1.12-9.1) for rs12255372. Although the OR was smaller when these two parameters were excluded from the analysis, there was a statistical significant result for both when compared with the controls.

BMI for cases and controls was compared for both SNPs after adjusting for all risk factors and exclusion of family history and FBS. There was significant difference at 0.05 level of significance (P <0.005) in the obese people only [OR= 3.5(1.3-9.5)].Statistical analysis for total cholesterol showed significant difference at 0.05 level of significance (P <0.005) between cases and controls [OR =13.9(1.6-122.67)] .Systolic blood pressure was significant OR =4.11(1.2-14.2) but diastolic was not significant OR=o.78(0.6-9.8), and the age was significant for 51-70 years OR =12.7(5.55-29.03),for 71-85years OR = 28(3.3-233.3).Here the gender was not significant no difference between males and females to have the disease ,the results of this analysis in table **(6).**

When studying the effect of each mutation alone after adjusting for other risk factors and exclusion of family history and FBS , for **rs7903146** it showed significant association with diabetes [OR=3.34(1.46-7.65)]. Comparing the following parameter for all participants (cases and controls), BMI for the obese people, total cholesterol, systolic blood pressure ,and age all showed statistical significant results as shown in Table 7. Statistical significant results were also observed indicating positive association with T2DM when testing the previously mentioned

parameters for the second mutation r**s12255372 [**OR= 5.83(2.18-15.56)], results are shown in Table 8.

We analyzed both mutations simultaneously (**rs7903146** and **rs12255372)** in the total population tested for all the parameters with possible development of T2DM. Although we observed significant results the OR was 3.6(1.08-11.98), much smaller than when each mutation tested alone, OR was 15.52(5.42-44.5) as shown in Table 9.

Allele specific PCR was used to detect the presence of SNPs on both alleles (homozygous genotype), or present in one allele only (heterozygous genotype), as shown in Table 10 and 11. Allele frequencies were calculated according to the following formula (116).

Two alleles are possible, M (mutated) or N (normal) that can combine to give the following genotypes MM, MN, NN, so the allelic frequency for the M allele will be:

***f*(M) = [(2 x M) + MN]/2×number of population**

The frequency for the N allele will be:

f(N) = [(2 x N) + MN]/2× number of population, so

f(M)+ *f*(N) = 1. (116)

The allele specific frequencies were 0.1039 for rs7903146 T allele in the controls and three folds higher 0.297in the cases. For the second mutation rs12255372 T allele, the allele specific frequency was 0.065 in controls and about four folds 0.2168 in cases .OR was calculated for both SNPs to assess its association with type 2diabetes as shown in the same Tables 10 and 11 before

and after adjustment for sex, age, BMI, systolic, diastolic blood pressure and total cholesterol as covariates, the results showed evidence of association of the rs7903146 T allele with T2D in agreement with previous studies. The OR for **heterozygou**s CT before adjustment was 5.77 (2.6-12.6),*P* value 0.000, and after adjustment OR was 3.5(1.37-8.8), *P* value 0.009. The OR For **homozygous** before adjustment was 3.1(1.0-9.3), *P* value 0.043 and after adjustment was 3.7

(1-13.5) *P* value 0.044.

For rs12255372 OR for **heterozygous** GT before adjustment was 5.12(2.3-11.8*) P* value 0.000, and after adjustment OR was 3.77(1.4-9.8) *P* value 0.007 giving significant results for both. For rs12255372 **homozygous** TT, it was significant before adjustment OR was 4.5(1-19) *P* value 0.047 but not significant after adjustment, OR was 3.6 (1-21) *P* value 0.15 thus it was not significant.

As a result, for all diabetic participants who carry a single mutation or both mutations in this research, Bethlehem took first place ,Ramallah Jerusalem then the North (Qalqelya, Tulkarem, and Nablus),as shown in Table 12.

Table 5: General characteristics for the study population (mean ± SD), N (%)

	total	Controls (n=77)	Cases (n=239)	*P*
Age	54.96 ± 11.4	42.99 ±8.12	58.66 ±9.59	0.000*
Gender Males Females	 111 (34%) 215 (66%)	 27(35.1) 50(64.9)	 84(33.7) 165(66.3)	0.830
BMI	30.2 ± 5.68	26.49 ±3.54	31.34±5.73	0.000
FBS	152.05 ±70.16	86.79 ±9.85	172.22±68.48	0.000
T. Cholesterol	181.19 ± 38.9	168 ±19.96	185.26±41.45	0.000
Family history Yes No	 220 (67.5%) 106 (32.5%)	 22(28.6) 55(71.4)	 198(79.5) 51(20.5)	0.000
BP (Sys)	127.34 ±17.77	118±11.25	130.22±18.44	0.000
BP (Dias)	78.64 ±10.41	76.49±7.93	79.07±11.01	0.350
Mutation 1 (rs7903146) Yes No	 126 (38.7%) 200 (61.3%)	 12(15.6 %) 65(84.4%)	 118(47.4%) 131(52.6%)	0.000
Mutation 2 (rs12255372) Yes No	 101 (31%) 225 (69%)	 7(9.1 %) 70(90.9%)	 94(37.8%) 155(62.2%)	0.000
Area 1(Bethlehem) 2(Ramallah) 3(Jerusalem) 4(controls=Different areas) 5(North west bank)	 58 (17.8%) 64 (19.6%) 60(18.4%) 77(23.4%) 67(20.4%)	 0 0 0 77(100%) 0	 58(23.3%) 64(25.7%) 60(24.1) 0 76(26.6%)	0.000

Table(6):*Adjusted for all risk factors in table, for rs7903146 and rs12255372# P < 0.05 include and without family history and Fasting blood sugar (FBS)

	Number	Crude OR (95% CI)	Adjusted OR (95%CI)* with FBS	Adjusted OR for all factors with family history except FBS	Adjusted OR for all factors without family history and FBS
Mutation rs7903146					
No (ref)	196	1	1	1	1
Yes	130	4.88 (2.51-9.48)#	4.31 (1.02-18.18)#	2.1(0.81-5.2)	3.0(1.24-7.24)
Mutationrs12255372					
No (ref)					
Yes	225	1	1	1	1
	101	6.065(2.68-13.74)	0.41(0.08-2.1)	3.3(1.12-9.1)	5.5(1.97-15.19)
BMI (kg/m2)					
18-24.9	52	1	1	1	1
25-29.9	111	1.78 (0.910-3.48)	2.26 (0.41-12.5)	1.12(.4-3.2)	1.24(0.49-3.2)
30-55	163	9.86 (4.55-21.32)#	7.83 (1.34-45.8)	2.5(0.8-7.3)	3.5(1.3-9.5)
Total cholesterol(mg/dL)					
40-239	275	1	1	1	1
240-400	51	19.01(2.6-140.7)	27.2(1.7-434.7)	12(1.2-122)	13.9(1.6-122.67)
Diastolic(mmHg)					
60-95	310	1	1	1	1
96-110	16	4.87(0.63-37.49)	0.66(0.04-11.8)	0.8(.06-9.3)	0.78(0.6-9.8)
Systolic(mmHg)					
90-139	242	1	1	1	1
140-210	84	15.8 (6.40-39.01)	1.64 (0.4-7.2)	2.5(0.7-8.7)	4.11(1.2-14.2)
Gender					
Male	111	1	1	1	1
Female	215	1.06(0.6-1.8)	0.5(.15-1.7)	1.05(0.45-2.5)	0.92(0.416-2.04)
Age(years)					
30-50 (1)	117	1	1	1	1
51-70 (2)	180	22.0(10.5-45.9)	16.97(5.2-56)	14.9(6-36.6)	12.7(5.55-29.03)
71-85(3)	29	36.2(4.8-275.3)	44.4(3.8-521.6)	29(3.4-250.6)	28(3.3-233.3)
Family history					
No	106	1	1	1	
Yes	220	9.7(5.4-17.4)	4.5(1.4-14.5)	6.1(2.6-14.2)	----------

Table (7):*Adjusted for all risk factors in table, for rs7903146 # P < 0.05.

	Number	**Crude OR (95% CI)**	**Adjusted OR (95% CI)* with Family history**	**Adjusted OR (95% CI)*without family history**
Mutation(1)rs7903146				
No (ref)	196	1	1	1
Yes	130	4.88 (2.51-9.48)#	2.28(0.93-5.58)	3.34(1.46-7.65)
BMI (kg/m2)				
18-24.9	52	1	1	1
25-29.9	111	1.78 (0.910-3.48)	0.99(0.36-2.72)	1.032(0.42-2.51)
30-55	163	9.86 (4.55-21.32)#	2.41(0.82-7.1)	3.81(1.45-10.03)
Total Cholesterol(mg/dL)				
40-239	275	1	1	1
240-400	51	19.01(2.6-140.7)	11.24(1.15-109.6)	12.12(1.42-103.2)
Diastolic(mmHg)				
Normal	310	1	1	1
Abnormal	16	4.87(0.63-37.49)	0.83(0.07-9.66)	0.85(0.73-9.82)
Systolic (mmHg)				
Normal	242	1	1	1
Abnormal	84	15.8 (6.40-39.01)	2.23(0.66-7.51)	3.57(1.095-11.62)
Gender				
Male	111	1	1	1
Female	215	1.06(0.6-1.8)	1.12(0.49-2.55)	1.01(0.47-2.15)
Age				
30-50 (1)	117	1	1	1
51-70 (2)	180	22.0(10.5-45.9)	15.25(6.21-37.4)	12.35(5.544-27.53)
71-85 (3)	29	36.2(4.8-275.3)	28.26(3.27-244.23)	23.97(2.93-196.34)
Family history				----------
No	106	1	1	
Yes	220	9.7(5.4-17.4)	7.41(3.26-16.82)	

Table (8):*Adjusted for all risk factors in table, for rs12255372# P < 0.05.

	Number	Crude OR (95% CI)	Adjusted OR (95% CI) *with Family history	Adjusted OR (95% CI)* without Family history
Mutation(2)rs12255372 No (ref) Yes	 225 101	 1 6.065(2.68-13.74)	 1 3.5(1.28-9.5)	 1 5.83(2.18-15.56)
BMI (kg/m2) 18-24.9 25-29.9 30-55	 52 111 163	 1 1.78 (0.910-3.48) 9.86 (4.55-21.32)#	 1 1.1(0.38-2.99) 2.3(0.8-6.74)	 1 1.19(0.48-2.97) 3.26(1.22-8.74)
Total Cholesterol(mg/dL) 40-239 240-400	 275 51	 1 19.01(2.6-140.7)	 1 12.77(1.33-123.1)	 1 15.57(1.83-32.8)
Diastolic(mmHg) Normal Abnormal	 310 16	 1 4.87(0.63-37.49)	 1 0.66(0.06-7.9)	 1 0.65(0.05-8.1)
Systolic(mmHg) Normal Abnormal	 242 84	 1 15.8 (6.40-39.01)	 1 2.53(0.73-8.8)	 1 4.4(1.29-14.99)
Gender Male Female	 111 215	 1 1.06(0.6-1.8)	 1 1.12(0.49-2.58)	 1 1.07(0.5-2.3)
Age 30-50 (1) 51-70 (2) 71-85 (3)	 117 180 29	 1 22.0(10.5-45.9) 36.2(4.8-275.3)	 1 15.77(6.46-38.54) 31.71(3.74-268.52)	 1 13.8(6.13-30.96) 33.99(4.15-278.4)
Family history No Yes	 106 220	 1 9.7(5.4-17.4)	 1 6.90(3.02-15.81)	----------

Table (9):* Adjusted for all risk factors in table. For interaction and combination between mutations **rs7903146 and rs12255372** together**,** # P < 0.05.
** (0): have not both mutations, (1): have single mutation either rs7903146or rs12255372, (2): have both mutations rs7903146 and rs12255372.

	Adjusted OR (95% CI) * with Family history	**Adjusted OR (95% CI) * without Family history**
Mutation(1)rs7903146+mutation(2)rs12255372 No (ref)(0)** Yes(1) (2)	 1 10.143.34-30.8) 2.21(0.65-7.5)	 1 15.52(5.42-44.5) 3.6(1.08-11.98)
BMI (kg/m2) 18-24.9 25-29.9 30-55	 1 1.27(0.43-3.77) 3.4(1.08-10.9)	 1 1.29(0.48-3.46) 4.4(1.53-12.8)
Total Cholesterol (mg/dL) 40-239 240-400	 1 13.3(1.21-147.7)	 1 15.1(1.064-139.0)
Diastolic(mmHg) Normal Abnormal	 1 0.56(0.042-7.5)	 1 0.57(0.042-7.85)
Systolic Normal Abnormal	 1 2.88(0.78-10.66)	 1 4.93(1.37-17.8)
Gender Male Female	 1 0.76(0.3-1.89)	 1 0.65(0.27-1.54)
Age 30-50 (1) 51-70 (2) 71-85 (3)	 1 19.3(7.5-49.9) 34.7(3.82-315.02)	 1 17.14(7.1-41.45) 33.53(3.85-291.8)
Family history No Yes	 1 5.46(2.3-13.01)	----------

Table (10): The genotype frequency of the r7903146 polymorphism in the *TCF7L2* gene

Genotype	Controls	T2DM	*P* value	Crude OR	Adjusted OR	*P*
CC	**65**	**131**	**reference**			
CT	**8**	**88**	**0.000**	**5.77 (2.6-12.6)**	**3.5(1.37-8.8)**	**0.009**
TT	**4**	**30**	**0.043**	**3.1(1.0-9.3)**	**3.7(1-13.5)**	**0.044**
Total	**77**	**249**				
Allele frequency						
Allele C	**0.896**	**0.7028**				
Allele T	**0.1039**	**0.297**				

Distribution of genotype and allele frequencies for polymorphism within the TCF7L2 gene **rs7903146** , and the analysis before and after incorporates sex, age, BMI ,systolic and diastolic blood pressure and total cholesterol as covariates, minimizing potential confounding (OR, odds ratio, CI, confidence interval).

Table 11: **The genotype frequency of the rs12255372 polymorphism in the *TCF7L2* gene**

Genotype	Control	T2DM	*P* value	Crude OR	Adjusted OR	*P* value
GG	**69**	**154**	**reference**			
GT	**6**	**76**	**0.000**	**5.12(2.3-11.8)**	**4.0(1.4-10)**	**0.009**
TT	**2**	**19**	**0.047**	**4.5(1-19)**	**3.6(1-21)**	**0.15**
Total	**77**	**249**				
Allele frequency						
Allele G	**0.935**	**0.783**				
Allele T	**0.065**	**0.2168**				

Distribution of genotype and allele frequencies for polymorphism within the TCF7L2 gene **rs12255372** and the analysis before and after incorporates sex, age, BMI ,systolic and diastolic blood pressure and total cholesterol as covariates, minimizing potential confounding (OR, odds ratio, CI, confidence interval)

Table (12): Distribution of TCF7L2 gene positive results for mutations **rs7903146** and **rs12255372** of Diabetes Mellitus in West Bank and Jerusalem, Palestine:

Area	**rs7903146**	**Ratio**	**rs12255372**	**Ratio**	Both mutations	**Ratio**
Bethlehem	31	31/58=53.4%	30	30/58=51.7%	15	15/58=25.8%
Ramallah	34	34/64=53%	24	24/64=37.5%	13	13/64=20%
Jerusalem	31	31/60=51.6%	18	18/60=30%	10	10/60=16.6%
North West Bank	22	22/67=32.8%	19	19/67=28.3%	7	7/67=10.4%

Chapter 4

Discussion

The aim of this case control study was to evaluate the association between TCF7L2 gene in T2DM. Two SNP's rs7903146C/T, rs12255372G/T in the TCF7L2 gene were investigated in diabetic patients and compared to a control group. RFLP PCR was performed using two restriction enzymes *RsaI* and *BseGI* to identify the presence of these two specific mutations in the study population. Furthermore, allele specific PCR was performed to substitute for DNA sequencing on one hand and to genotype the TCF7L2 gene as homo or heterozygous. We used SPSS v.21 to compare the results obtained between the case and control groups.

The results of the RFLP showed the presence of significant association at 0.05 level of significance between the two investigated single nucleotide polymorphisms and T2DM. The details of the results are shown in Tables 10 and 11. This is the first study that evaluated the association of two mutations with T2DMin this country. A study conducted by Abdeen et al. in 2010 in Al-Quds University (99) on rs7903146C/T reported similar results as compared to ours. We used allele specific PCR to genotype the gene investigated instead of the sequencing analysis they used. Previous studies evaluated the association of both mutations as used by us conducted by researchers in the US, Poland, Amish population in the US ,Finnish, and French have similar results for rs7903146 C/T, rs12255372 G/T both of these SNPs are associated with T2DM (90,95,96,117,118,120).

The study performed on the association of TCF7L2 gene and T2DM on the Palestinian population by Abdeen et al., reported an association between the**rs7903146** T allele and T2DM

as reported in this study. The population selected for Abdeen study was restricted to one geographic area, Ramallah, Palestine. Their samples were collected from the UNRWA clinics in the Ramallah area. Our study is more comprehensive and more representative of the Palestinian population and the different geographical regions of Palestine. Furthermore, our study addressed the two strongest and most commonly involved alleles in T2DM, while Abdeens study addressed only one allele.

A study conducted on Israeli Ashkenazi Jewish population on 2010 (121).This study found a strong independent association between HNF4 and WFS1 alleles with T2DM in this ethnic group. They also evaluated **rs7903172** T allele and reported a weak association with T2DM contrary to results obtained in our study. The allele frequency for this allele was high than that reported by the Israeli study indicating that this allele is strongly associated with T2DM in Palestinian population as compared to weak association among the Ashkenazi Jews.

In a Mexican study conducted by Parra EJ, in 2007(114) reported significant association between rs12255372 while SNP rs7903146 showed similar trends, but its association with T2D is not as strong as rs12255372. This may reflect geographic distribution regarding the polymorphism of TCF7L2 gene.

Although most of the literature published to evaluate the polymorphism of the TCF7L2 genes relied on sequencing analysis, we adopted the allele specific PCR method (122,123,124,125). Allele specific PCR is a reliable technique that has high specificity and sensitivity (126,127,128,129,130). In addition, it is cost effective, rapid and not labor intensive. The results obtained by this method in this study were similar to results obtained by others who used the DNA sequencing (99,114, 90,

95, 96, 117, 118, 120). The results obtained by this method accurately identified the genotypes and nature of polymorphism of the TCF7L2 gene among the Palestinian population tested.

The nature of TCF7L2 gene polymorphism differs from one geographic area and another. In China for example, they did not detect association of the risk allele (rs7904146 T and rs12255372 T) with type 2 diabetes as detected in our study. These risk alleles were found to be rare with low frequencies in the Chinese population (131). A possible explanation for this result could be attributed to differences in the ethnic background among the Chinese population or the effects of environmental factors, such as life-style as well as geographical distribution of these alleles.

By using RFLP –PCR with exclusion family history and FBS because both are very significant and strongly related to type 2 diabetes so if both included in analysis the association between two mutations and the disease will be less significant thus both were excluded. The same for **rs7903146** after adjusting for other risk factors ,age, sex , BMI, total cholesterol, and blood pressure it showed significant association with diabetes OR=3.34(1.46-7.65, as shown in table (7).

The allele specific PCR for **rs12255372 T** allele has a higher frequency in the patients with T2D than the controls as shown in Table 11. It was noteworthy that the heterzygote genotype for this allele was statistically significant before and after adjustment for the confounding factors (risk factors, age, sex, BMI, total cholesterol, and blood pressure). Contrary to that, it was found that for the homozygous genotype of this allele was statistically significant before adjustment but not statistically significant after adjustment for the confounding factors. This can be due to scarcity

of this mutation among the control group. Similar results have been reported by a Mexican study but on the **rs12255346 T** allele (114).

The interaction between mutations rs7903146 and rs12255372 on the *TCF7L2* gene and T2DM combined had an OR= 3.6(1.08-11.98) but the OR was 15.52(5.42-44.5) when these mutations were considered singly as shown in Table9. The differences in the OR in these situations may be explained by the considering that every mutation works independently with distinct mechanism and it is not common to find both mutations together in the same individual. The exact mechanisms, by which genetic variation within the intron of the TCF7L2 gene that confer susceptibility to Type 2 diabetes remain to be elucidated. Genetic variations near the 3′ end of the TCF7L2 gene may affect the action of TCF7L2, through the regulation of alternative splicing (120).

There was difference in the mutation distribution between south to north Palestine, mainly Bethlehem (south) occupied the first place in diabetic patients who carry the mutations in TCF7L2 gene, many reasons may interact like high trend of positive family history for diabetes among the study population in the south may be due to relative marriage contributing to the perpetuation of genetic disorders or adopting of adverse health behaviors like eating habits, smoking and other environmental factors that differ from one geographic region to another in Palestine, which contribute to development of diabetes disease when interacting with a susceptible gene.

A limitation of this study could be that the number of the control participants was relatively small and may have caused some bias when comparing age and gender, blood pressure and cholesterol, these confounding factors have been adjusted in the statistical analysis to obtain a more real picture of the association between T2DM and TCF7L2 gene.

As a conclusion, a significant association of the TCF7L2 variant with type 2 diabetes risk was observed in Palestinians for both polymorphisms rs7903146 and rs12255372, but rs7903146 was more significant than rs12255372 between Palestinians. As known, the strongest association with T2DM risk in most reported studies is with rs7903146 that shows a stronger association with type 2 diabetes than rs12255372.

Chapter 5

Incretin-Based Therapy in the Prediabetic Stage

Pre-diabetes individuals who are at high risk for developing Type 2 diabetes , many causes are associated for developing T2DM such as family history, overweight or obese, women who have had gestational diabetes with high birth weight babies , people with IGT or IFG, they have lost 80% of their β-cell function, they have an approximate 10% incidence of diabetic retinopathy and genetics factors that multiple genetic defects at different loci will be found that confer varying degrees of predisposition to Type 2 diabetes .The clinical implications of these findings for the treatment of Type 2 diabetes are that the physician must intervene early (132,133,134).

Variety of pharmacologic treatment options management of patients with pre-diabetes could be used after using some of the strategies that includes medical nutrition therapy to achieve weight loss in individuals who are overweight or obese, appropriately prescribed physical activity, avoidance of tobacco products, and stress reduction (135,136). The goals of early (pre-diabetic) glucose-directed therapies are to normalize glucose levels, prevent or delay progression to diabetes, prevent microvascular complications, and modify other risk factors such as obesity, hypertension, and dyslipidemia (134).

There are many hypoglycemic drugs that can be used such as the oral diabetes drugs like **metformin** (glucophage) which has a biguanide that improves the ability of insulin to suppress excess hepatic glucose production in both the fasting and postprandial states (137). **Acarbose** (class – alpha-glucosidase inhibitors) an alpha-glucosidase inhibitor, works by delaying carbohydrate absorption from the intestine also used for high risk of diabetes (138). **Pioglitazone or rosiglitazone** (class – thiazolidinediones [TZDs]) are effective in preventing T2DM in 62% to 72% of high-risk patients,

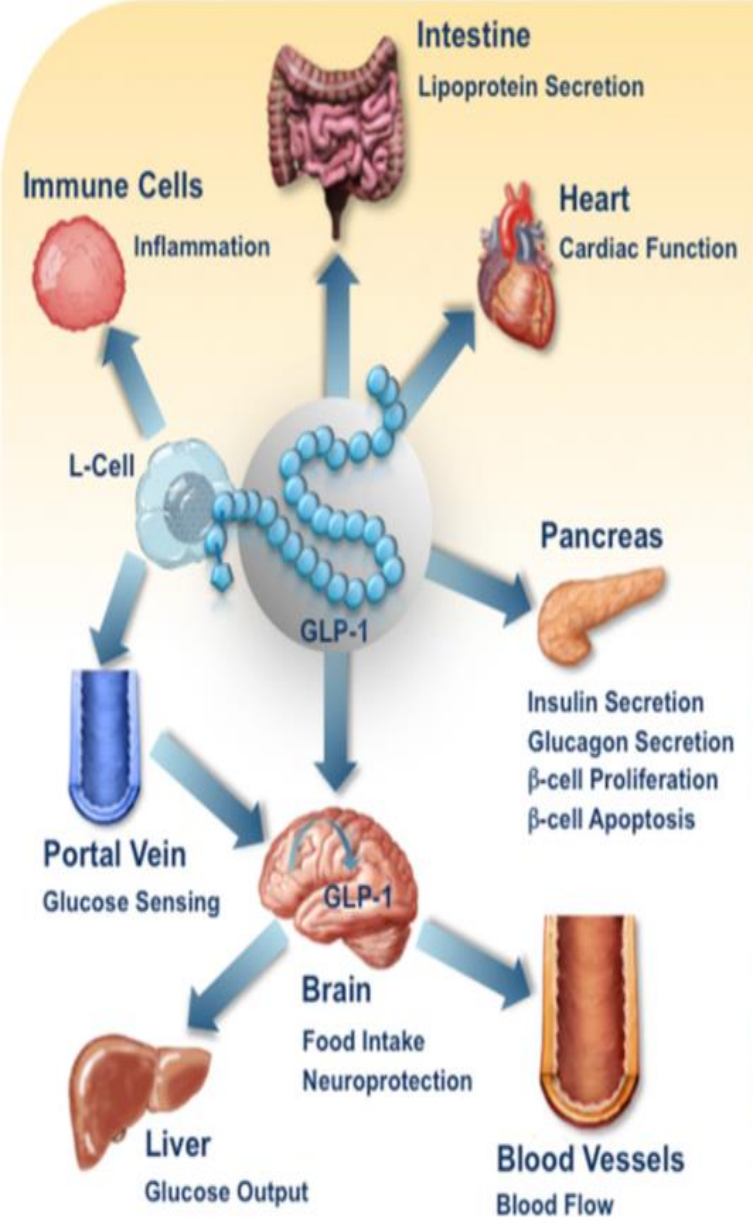
TZDs increase peripheral insulin sensitivity, with the benefit of increased HDL cholesterol and decreased LDL cholesterol (139). **Exenatide** (class – glucagon-like peptide-1 [GLP-1] inhibitor or **Byetta)** as a novel treatment option for type 2 diabetes mellitus it belongs to the group of incretin mimetics**:** augments pancreas response, increases insulin secretion in response to eating meals**,** suppresses pancreatic release of glucagon in response to eating**,** has a subtle yet prolonged effect to reduce appetite, promote satiety via hypothalamic receptor (140) .

5.1 Genetic Variants in TCF7L2 Gene and Incretin Effect

The TCF7L2 polymorphisms are thought to make a greater contribution to the development of Type 2 diabetes than other genetic markers (141). The TCF7L2 variants mainly in the Two intronicTCF7L2 polymorphisms, rs7903146 C>T and rs12255372 G>T, have been the strongest and most consistent predictors of increased Type 2 diabetes risk because they are associated with a reduced incretin effect (142,143,144).

Incretins are gut hormones that potentiate insulin secretion after meal ingestion in a glucose-dependent manner. The two best-studied incretins, glucose-dependent insulinotropic polypeptide (GIP) secreted from the L-cells of the distal ileum and glucagon-like peptide-1 (GLP-1) secreted from the K-cells in the duodenum and jejunum. After secretion, incretins are rapidly degraded due to the action of dipeptidyl peptidase-4 (DPP-4), a ubiquitous enzyme found on the surface of epithelial and endothelial cells but also found in plasma (145).

There are many biological actions of incretins related to pancreas mainly for GLP-1 that increases insulin and inhibits glucagon secretion in a glucose-dependent manner, GLP-1 also increases insulin synthesis, confers glucose sensitivity to glucose-resistant beta cells, stimulates beta-cell proliferation and neogenesis, and inhibits beta cell apoptosis, also there are another physiological actions for GLP-1 in other organs in the body, as shown in Figures 16 (146).



Physiological Actions of GLP-1

Physiology	Impairment of GLP-1 action
Fasting Glucose	Increased
Glucose-Dependent Insulin Secretion	Decreased
Glucagon Secretion	Increased
β-cell Signal Transduction	Abnormal
β-cell Apoptosis	Increased
β-cell Proliferation	Decreased
Islet Size	Decreased
Food Intake	Increased
Energy Expenditure	Increased
Locomotion	Increased
Body Weight	Increased
Portal Glucose Sensing	Decreased
Postprandial Lipid Metabolism	Decreased
CNS Control of Blood Flow	Decreased
Cardiovascular Function	Decreased
CNS Stress Response	Increased
Susceptibility to Neural Injury	Increased
Immune System	Decreased
Gastric Emptying	Normal
Susceptibility to Pancreatitis	Normal

Figure 16: Physiological Roles of Endogenous GLP-1**.**Campbell and Drucker, Pharmacology, Physiology, and Mechanisms of Incretin Hormone Action, Cell Metabolism (2013), http://dx.doi.org/10.1016/j.cmet.2013.04.008

Now a day incretin-based therapies offer a new approach for the management of type 2 diabetes, with a mechanism of action distinct from any existing class of glucose-lowering agents. These drugs improve the body's ability to control blood glucose by increasing active concentrations of glucagon-like peptide-1 (GLP-1).Two approaches have been used to enhance the action of GLP1. First, incretin mimetics (exenatide) or analogues (liraglutide) act as agonists at the GLP-1 receptor to mimic the effect of endogenous GLP-1, and dipeptidyl peptidase IV (DPP-4) inhibitors, which prevent metabolism of the native peptide and extend its activity (147).

According to our research study, using the incretins for non-diabetic subjects with SNPs in the TCF7L2 gene (TT or TC at rs7903146) or (TT or TG at rs12255372) might even be disease-modifying agents that have the potential to delay the onset or slow the progression of diabetes (148).

As known, TCF7L2 encodes a transcription factor involved in Wnt signaling, important for the development of the pancreatic β-cells and for β-cell survival. Therefore, TCF7L2 variants may underlie the loss of incretin effect (148),Human non-diabetic subjects with SNPs in the TCF7L2 gene (TT or TC at rs7903146) or (TT or TG at rs12255372) exhibit reduced insulin secretion in response to oral but not intravenous glucose, with no differences in circulating GLP-1 or GIP levels or insulin sensitivity, consistent with the notion that reduction in TCF7L2 impairs beta cell incretin responsivity (149).Non-diabetic carriers of the diabetes-associated TCF7L2 variant allele exhibit normal beta cell function in response to exogenous GLP-1 infusion during hyperglycemic clamp (150). In contrast, insulin secretion in response to oral glucose or a mixed meal was reduced in non-diabetic carriers of rs7903146 or rs12255372, and the insulinotropic response to exogenous GLP-1 and GIP was impaired during a hyperglycaemic clamp (151).

Therefore, incretin-based therapy is a valuable add-on to the therapeutic spectrum for pre-diabetes that offers the possibility of targeting many pathophysiological abnormalities associated with the disease, but this needs to be proven by clinical trials**.**

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5. Appendix

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
1	B	52	F	23	100/70	211	212	Y	P	P
2	B	56	F	25	110/60	258	191	Y	P	P
3	B	63	F	23	120/80	196	200	Y	P	P
4	B	50	F	32	130/80	152	200	Y	P	N
5	B	46	M	30	120/80	140	187	Y	P	P
6	B	61	F	34	120/80	170	234	N	P	N
7	B	68	F	24	100/70	129	196	Y	N	P
8	B	47	F	40	130/80	140	210	Y	P	N
9	B	67	F	26	138/88	104	247	N	P	P
10	B	44	F	34	120/80	233	196	Y	P	P
11	B	80	M	24	130/80	94	206	Y	N	N
12	B	81	M	28	138/80	158	190	Y	P	P
13	B	69	F	33	130/60	139	150	Y	P	N
14	B	59	M	29	150/90	223	190	Y	N	N
15	B	59	F	37	110/50	83	137	Y	P	P
16	B	51	F	29	150/90	170	182	Y	N	N
17	B	80	M	36	130/60	162	103	Y	P	P
18	B	46	M	34	150/100	112	206	Y	N	N
19	B	60	F	28	130/180	151	210	Y	N	P
20	B	62	F	30	110/80	124	130	Y	N	N
21	B	67	F	29	110/80	222	168	Y	P	N
22	B	64	F	28	130/70	129	192	N	N	N
23	B	57	F	34	120/80	146	174	Y	P	P
24	B	74	F	36	120/80	180	198	Y	P	P
25	B	57	F	32	110/72	105	166	Y	P	P
26	B	50	F	24	90/60	305	202	Y	N	P
27	B	67	F	40	150/70	191	204	Y	N	P

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
28	B	78	F	31	130/60	118	152	Y	N	P
29	B	61	M	29	138/88	126	181	Y	P	N
30	B	51	F	44	130/82	304	143	Y	N	N
31	B	58	F	32	130/70	136	202	Y	P	N
32	B	63	F	45	130/80	134	192	Y	P	N
33	B	56	F	44	110/70	188	162	Y	P	N
34	B	53	F	25	150/90	215	194	Y	P	P
35	B	67	M	41	150/60	347	269	Y	N	P
36	B	71	F	30	120/80	136	65	N	N	N
37	B	66	F	42	130/80	254	238	Y	N	N
38	B	48	M	29	120/60	120	196	Y	N	N
39	B	46	F	29	110/80	170	148	Y	P	N
40	B	43	F	34	120/80	183	211	N	P	P
41	B	43	F	29	110/70	140	170	Y	N	N
42	B	68	M	28	138/70	203	153	N	P	N
43	B	72	F	35	180/90	267	220	N	P	P
44	B	56	M	26	120/70	97	183	Y	N	N
45	B	53	M	26	130/84	118	150	Y	P	P
46	B	64	F	36	150/80	296	190	Y	N	N
47	B	50	F	34	120/70	108	178	Y	P	P
48	B	52	F	33	100/60	159	287	Y	P	P
49	B	67	F	38	120/70	170	255	Y	P	N
50	B	59	F	38	130/80	253	278	Y	N	P
51	B	63	F	27	130/80	164	212	Y	N	P
52	B	69	F	28	138/80	239	230	Y	N	P

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
53	B	74	M	28	130/80	127	217	Y	N	P
54	B	75	M	34	134/70	134	188	Y	P	P
55	B	68	F	32	150/110	120	153	Y	N	P
56	B	63	F	37	130/80	191	295	Y	P	P
57	B	58	F	43	128/88	169	208	Y	P	N
58	B	55	F	39	120/86	233	135	Y	P	P
59	R	55	F	24.9	135/88	237	180	T	N	P
60	R	70	M	34.4	134/91	230	231	Y	N	P
61	R	60	F	34	169/87	257	158	Y	P	N
62	R	56	M	27.4	150/85	236	244	Y	N	P
63	R	73	F	33.5	152/79	240	221	N	P	P
64	R	64	F	28.4	120/80	234	248	N	P	P
65	R	56	F	30.8	127/65	131	183	Y	P	P
66	R	64	F	33	144/75	85	178	Y	N	P
67	R	73	M	27.6	122/66	87	128	Y	N	P
68	R	48	F	34	122/66	281	230	Y	P	P
69	R	62	F	30	133/77	233	114	Y	N	P
70	R	66	F	33	148/79	100	216	Y	N	P
71	R	66	F	31.6	143/82	101	145	Y	N	P
72	R	73	F	27	149/87	128	219	Y	P	N
73	R	42	M	24.9	146/89	136	142	Y	N	N
74	R	54	M	33.6	123/87	148	216	Y	P	N
75	R	46	M	31.3	116/67	94	240	Y	P	N
76	R	64	M	28	122/70	139	133	Y	P	P
77	R	54	M	29.6	144/90	68	127	Y	N	P
78	R	39	F	36	130/85	140	194	Y	P	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
79	R	63	F	44	126/72	164	201	Y	N	N
80	R	51	F	32	134/90	250	161	Y	P	P
81	R	47	M	36	112/73	309	149	Y	N	N
82	R	62	M	26	129/70	145	173	Y	N	P
83	R	75	F	33	90/60	118	167	Y	N	P
84	R	55	F	25	120/80	143	240	Y	P	P
85	R	61	M	40	100/50	165	111	Y	N	N
86	R	63	F	46	100/70	123	318	Y	N	N
87	R	43	M	33	110/70	156	216	Y	P	N
88	R	36	F	24	130/88	220	180	Y	P	N
89	R	72	M	26	130/80	135	234	N	N	P
90	R	66	M	28	134/83	197	190	Y	N	N
91	R	54	M	29	113/65	159	186	N	P	P
92	R	57	M	29	146/79	168	171	Y	P	N
93	R	48	F	23	109/68	148	151	Y	N	P
94	R	45	F	29	90/50	173	166	Y	P	N
95	R	67	F	47	160/70	261	226	Y	N	P
96	R	53	F	33	90/60	156	249	Y	N	N
97	R	53	F	37	110/55	403	225	Y	P	P
98	R	58	F	35	100/70	255	128	Y	N	P
99	R	79	M	23	120/80	150	113	Y	N	N
100	R	65	F	30	110/70	165	131	Y	N	N
101	R	57	F	33	100/60	159	265	Y	N	N
102	R	50	F	27	90/60	121	212	N	N	P
103	R	60	F	33	100/60	257	176	Y	N	P
104	R	44	M	23	100/60	162	174	Y	P	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
105	R	69	F	33	120/60	116	207	Y	N	P
106	R	54	M	28	140/80	142	120	Y	P	N
107	R	51	M	30	204/110	155	218	Y	N	P
108	R	76	M	20	85/50	434	214	Y	N	N
109	R	59	M	36	130/80	170	243	Y	P	P
110	R	74	M	31	95/50	114	162	Y	N	P
111	R	68	M	30	135	200	190	Y	N	P
112	R	45	M	28	90/60	109	172	Y	N	N
113	R	61	M	29	160/90	220	170	Y	N	P
114	R	38	M	32	120/89	153	191	Y	N	P
115	R	62	M	31	160/90	128	206	N	N	N
116	R	73	M	39	100/70	136	182	Y	N	N
117	R	60	F	39	130/69	206	180	Y	P	N
118	R	50	F	25	100/70	90	186	Y	N	N
119	R	75	F	32	140/90	129	268	Y	P	P
120	R	43	M	37	167/92	238	228	Y	N	P
121	R	52	F	31	130/85	280	238	Y	N	N
122	R	60	M	30	140/90	117	243	Y	N	P
1537	J	55	F	33	140/90	155	188	N	P	P
1538	J	64	F	39	126/72	157	169	N	P	P
1539	J	50	F	34	150/84	248	139	Y	N	P
1540	J	69	M	29	130/84	128	202	Y	N	P
1541	J	65	M	26	130/85	256	222	Y	N	P
1542	J	71	F	27	158/72	219	160	Y	P	P
1543	J	51	F	36	140/90	126	202	Y	P	P
1544	J	64	F	30	130/82	80	189	Y	N	N
1545	J	57	F	27	150/86	159	119	Y	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
1546	J	57	F	36	120/86	169	182	Y	P	P
1547	J	53	F	31	120/82	179	248	Y	N	N
1548	J	45	F	34	140/100	127	192	Y	P	N
1549	J	57	F	32	150/96	120	159	Y	N	P
1550	J	48	F	30	110/82	174	216	Y	N	P
1551	J	55	M	31	130/82	110	205	N	P	P
1552	J	68	F	31	130/100	129	158	Y	P	N
1553	J	63	F	33	160/80	114	205	Y	P	N
1554	J	56	F	31	100/72	187	203	Y	N	P
1555	J	58	F	48	140/72	268	225	Y	N	N
1556	J	61	F	36	170/92	235	219	Y	P	P
1557	J	63	F	28	164/92	219	185	Y	N	P
1558	J	62	F	35	130/180	67	161	N	N	N
1559	J	61	M	30	150/68	137	189	Y	N	P
1560	J	63	F	34	142/86	153	151	Y	N	P
1561	J	69	F	27	120/164	140	136	N	P	P
1562	J	63	M	26	140/92	167	205	Y	P	P
1563	J	60	F	32	138/82	316	249	N	N	P
1564	J	60	F	41	138/86	254	154	N	N	N
1565	J	45	F	32	140/90	101	80	Y	N	N
1566	J	66	F	36	130/68	142	221	Y	P	P
1567	J	58	F	37	110/166	90	100	Y	N	N
1568	J	63	M	29	130/74	222	152	Y	N	N
1569	J	52	M	32	130/70	225	176	Y	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-choles.	Family History	rs12255372	rs7903146
1570	J	63	F	26	130/70	207	227	Y	P	P
1571	J	58	F	38	120/86	126	151	N	N	N
1572	J	53	F	39	140/92	116	233	Y	P	N
1573	J	64	F	32	110/76	132	180	N	N	P
1574	J	49	F	27	126/78	176	229	Y	N	P
1575	J	60	F	30	110/66	475	148	Y	P	N
1576	J	62	F	33	144/96	196	185	Y	N	N
1577	J	67	M	42	146/84	109	130	Y	N	N
1578	J	52	M	25	110/76	161	102	Y	N	N
1579	J	63	F	31	156/100	95	171	Y	N	N
1580	J	50	M	37	142/100	144	214	Y	N	N
1581	J	68	F	34	142/86	250	159	Y	P	N
1582	J	78	M	30	132/88	149	185	Y	P	P
1583	J	68	M	30	170/100	140	89	Y	N	N
1584	J	45	F	28	130/80	119	179	N	N	P
1585	J	51	M	25	140/82	123	117	Y	P	P
1586	J	60	F	40	136/88	108	202	N	N	N
1587	J	63	F	28	130/80	125	236	Y	P	P
1588	J	76	F	46	130/70	126	176	Y	P	P
1589	J	60	F	35	140/90	186	139	Y	P	P
1590	J	60	F	27	120/72	112	132	Y	N	N
1591	J	53	M	40	140/88	264	178	Y	N	N
1592	J	72	M	26	160/80	124	177	N	P	N
1593	J	42	F	39	126/74	81	187	Y	N	N
1594	J	71	F	32	180/90	125	211	N	N	N
1595	J	59	F	38	120/82	143	123	Y	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
1596	J	56	F	51	120/76	104	226	Y	P	N
C1	D	44	M	25	110/84	85	176	N	N	N
C2	D	36	F	22	122/81	87	150	N	N	N
C3	D	40	M	26	115/75	90	180	N	N	N
C4	D	44	M	24	118/86	79	161	N	N	N
C5	D	42	F	25	112/78	82	161	N	N	N
C6	D	45	F	23	118/84	88	145	N	N	N
C7	D	47	F	23	110/77	81	150	N	N	N
C8	D	45	F	26	124/82	85	148	N	N	P
C9	D	41	F	26	116/80	89	155	N	N	N
C10	D	37	F	27	120/81	92	165	N	N	N
C11	D	32	F	26	90/60	60	156	N	N	N
C12	D	45	M	27	120/80	88	166	N	N	N
C13	D	61	M	34	140/90	74	129	N	N	N
C14	D	32	F	27	110/80	70	156	N	N	N
C15	D	73	F	34	126/62	91	147	N	N	N
C16	D	37	F	24	110/70	87	140	N	N	N
C17	D	32	F	22	100/60	84	150	N	N	N
C18	D	50	F	27	110/70	90	156	N	N	N
C19	D	51	F	22	108/70	86	170	N	N	N
C20	D	48	F	21	110/60	85	160	N	N	N
C21	D	34	M	25	128/77	85	194	N	N	N
C22	D	36	M	24	120/80	85	173	N	N	N
C23	D	37	F	23	109/76	80	139	N	N	N
C24	D	49	M	29	129/79	85	152	N	N	N
C25	D	39	F	25	113/75	86	145	N	N	N
C26	D	41	F	24	111/71	99	192	N	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
C27	D	58	F	26	110/70	80	155	N	N	N
C28	D	38	M	25	108/72	79	141	N	N	N
C29	D	40	M	23	120/80	83	145	N	N	N
C30	D	42	M	28	122/84	75	200	N	N	N
C31	D	36	F	28	123/81	80	149	N	N	N
C32	D	35	F	24	117/75	78	143	N	N	N
C33	D	44	M	26	115/85	77	160	N	N	N
C34	D	45	M	27	112/77	95	180	N	N	N
C35	D	34	M	22	125/79	77	177	N	N	N
C36	D	37	M	24	119/79	84	222	N	N	N
C37	D	32	F	24	113/75	82	134	N	N	N
C38	D	54	F	25	130/82	89	183	N	N	N
C39	D	50	F	23	122/76	83	168	N	N	N
C40	D	42	F	31	109/69	88	135	N	P	N
C41	D	41	F	26	100/70	89	186	N	N	P
C42	D	33	F	21	100/60	82	160	N	N	P
C43	D	35	F	25	121/84	78	155	N	N	N
C44	D	47	M	30	115/74	99	172	N	N	N
C45	D	33	F	26	120/80	85	178	N	N	N
C46	D	38	F	25	121/81	86	170	N	N	N
C47	D	46	F	24	106/70	81	166	N	N	N
C48	D	40	M	25	120/82	75	150	N	N	N
C49	D	41	F	24	128/80	79	183	N	N	N
C50	D	47	F	23	112/77	76	164	N	N	N
C51	D	55	M	25	115/70	86	157	N	N	N
C52	D	56	F	25	123/78	89	190	N	N	N
C53	D	43	M	27	126/80	91	187	N	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
C54	D	42	M	28	105/74	93	167	N	N	N
RF1	D	50	F	28	146/180	108	162	Y	P	P
RF2	D	48	F	27	120/72	98	183	Y	N	N
RF3	D	37	F	32	110/70	90	190	Y	N	N
RF4	D	43	F	36	126/70	102	162	Y	N	N
RF5	D	59	M	29	100/60	95	139	Y	P	P
RF6	D	62	M	28	124/75	115	185	N	P	P
RF7	D	41	F	30	120/80	100	175	Y	N	N
RF8	D	48	M	23	120/80	75	163	Y	P	P
RF9	D	43	F	22	115/70	80	180	Y	N	P
RF10	D	34	F	24	110/80	88	179	Y	P	P
RF11	D	53	F	37	160/100	91	165	Y	N	N
RF12	D	36	M	29	120/65	77	188	Y	N	N
RF13	D	33	F	32	127/71	77	171	Y	P	N
RF14	D	45	M	30	125/87	88	183	Y	N	N
RF15	D	39	F	26	125/88	90	210	Y	N	P
RF16	D	42	M	30	115/70	110	190	Y	N	N
RF17	D	37	F	28	117/73	102	178	Y	N	N
RF18	D	38	F	29	105/94	92	153	Y	N	N
RF19	D	36	F	23	105/76	83	180	Y	N	P
RF20	D	49	F	35	142/64	85	213	Y	N	N
RF21	D	39	F	30	124/78	92	200	Y	N	N
RF22	D	56	F	32	130/85	90	199	Y	N	N
RF23	D	40	M	29	146/90	123	196	Y	N	P

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
Q1	N	53	M	30	141/90	157	190	Y	N	N
Q2	N	48	F	27	126/72	246	137	Y	N	N
Q3	N	63	F	36	150/84	254	220	Y	N	N
Q4	N	49	M	31	130/84	124	204	N	N	N
Q5	N	55	F	34	130/84	161	121	N	N	N
Q6	N	51	F	32	158/74	177	250	N	N	N
Q7	N	55	F	30	140/90	118	161	Y	N	N
Q8	N	53	M	31	130/82	110	209	Y	N	N
Q9	N	61	F	31	150/86	112	209	N	N	N
Q10	N	56	F	33	120/86	270	223	N	N	N
Q11	N	62	F	31	120/82	217	183	Y	N	N
Q12	N	67	F	46	142/95	135	190	Y	N	N
Q13	N	58	F	36	151/96	142	138	Y	N	N
Q14	N	43	M	28	111/83	320	247	Y	N	N
Q15	N	56	F	35	130/82	102	70	N	N	N
Q16	N	50	F	30	129/79	95	221	Y	N	N
Q17	N	56	M	34	162/100	220	175	Y	P	P
Q18	N	62	F	27	101/72	128	149	Y	N	N
Q19	N	58	F	25	140/80	130	185	Y	N	N
Q20	N	65	F	26	169/93	330	146	N	P	N
Q21	N	61	F	32	165/90	107	132	Y	N	N
Q22	N	66	M	41	130/80	94	172	Y	N	N
Q23	N	66	M	32	152/70	158	250	Y	N	N
Q24	N	49	F	36	144/88	140	91	N	N	N
Q25	N	61	F	37	119/64	126	116	N	N	N
Q26	N	58	M	29	140/96	122	233	Y	N	N
Q27	N	51	F	32	136/82	184	136	Y	N	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
Q28	N	40	M	26	140/84	266	187	Y	N	N
Q29	N	57	M	22	138/90	80	178	Y	N	N
Q30	N	54	F	24	130/68	141	123	Y	N	N
Q31	N	69	M	38	110/69	126	200	N	N	N
Q32	N	70	F	39	125/74	217	162	Y	N	N
Q33	N	58	F	32	127/70	78	188	N	N	N
Q34	N	46	F	26	112/77	248	157	Y	N	N
Q35	N	67	M	29	164/89	174	167	N	N	N
Q36	N	36	F	25	104/70	293	155	Y	N	N
Q37	N	40	F	19	125/82	217	152	Y	P	P
Q38	N	42	F	21	133/85	205	205	N	N	N
Q39	N	74	F	25	130/70	125	190	Y	N	N
Q40	N	58	F	30	120/86	177	218	N	P	N
Q41	N	39	M	24	142/92	131	160	Y	N	N
Q42	N	76	F	40	110/77	185	201	N	N	N
Q43	N	48	M	33	123/78	221	237	Y	N	P
Q44	N	50	F	24	112/66	169	159	Y	N	N
Q45	N	60	F	31	146/96	151	154	N	N	N
Q46	N	47	F	37	147/83	169	202	Y	N	N
Q47	N	51	M	34	156/101	116	156	N	P	N
Q48	N	66	F	31	147/90	217	222	Y	N	N
Q49	N	55	F	32	131/81	247	222	Y	P	N
Q50	N	35	F	29	130/87	233	170	N	P	P
Q51	N	60	F	22	107/78	171	239	N	N	N
Q52	N	63	F	20	115/83	172	162	Y	N	P
Q53	N	57	F	24	127/90	385	179	Y	N	N
Q54	N	73	M	27	121/84	124	209	Y	P	N

Number	Area	Age	Gender	BMI	BP	FBS	T-Choles.	Family History	rs12255372	rs7903146
Q55	N	61	F	26	134/88	468	221	N	P	P
Q56	N	53	F	27	126/76	117	145	Y	N	P
Q57	N	45	F	22	115/66	156	154	N	P	N
Q58	N	41	M	23	140/85	111	240	Y	P	N
Q59	N	52	M	22	144/82	230	172	Y	N	P
Q60	N	65	M	25	137/81	118	189	N	N	N
Q61	N	66	M	24	125/84	96	210	N	N	N
Q62	N	63	M	24	130/70	160	222	Y	N	P
Q64	N	63	M	25	130/75	174	155	Y	N	P
Q65	N	55	M	25	111/69	153	199	N	N	N
Q66	N	54	M	22	141/86	164	138	Y	N	N
Q67	N	73	F	27	120/86	180	162	Y	N	N
Q68	N	70	F	29	148/81	102	150	Y	N	N

Table 4: Demographics and related information for the participants of this project.
B: Bethlehem, R: Ramallah, J: Jerusalem, D: difference areas. C: normal control, RF: risk factors control. BMI: body mass index. FBS: fasting blood sugar, T.Choles.: total cholesterol.
P and N are the results for positive and negative for the T-allele in cases and controls for the two mutations (rs7903146, rs12255372)

Birziet University

Faculty of Nursing Pharmacy and Health Professions

Master Program in Clinical Laboratory Science

Questionnaire

Project title: Detection of polymorphism in TF7L2 gene and association with type 2Diabetes mellitus

Name and number, الأسم/الرقم:_____

Age / العمر :_____ Gender / الجنس :_____

Weight / الوزن :_____ Height / الطول :_____

Family History / تاريخ العائلة

Number of diabetic family members / أفراد العائلة المصابين بالسكري :_____

Relationship / صلة القرابة:_____

Clinical Tests / الفحوصات :

Blood pressure /ضغط الدم :_____

Fasting blood sugar / نسبة السكر بعد الصيام :_____

Total cholesterol / الكوليسترول الكلي :_____

Patient's consent / موافقة المريض :

I agree to participate in this study / أوافق على المشاركة بهذه الدراسة :_____