The Islamic University–Gaza

Research and Postgraduate Affairs

Faculty of Science

Master of Medical Technology

الجامعـــــة الإســلاميـة– غزة

شئون البحث العلمي والدراسات العليا

كليــة: العلوم

ماجستير تحاليل طبية

Candidate Gene Polymorphisms and Risk of Type-2 Diabetes Mellitus in Gaza Strip

التعدد الشكلي لجينات ذات صلة بخطر الاصابة بمرض السكري من النوع الثاني في قطاع غزة

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Molecular Biology

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Biological Sciences

April /2017

إقـــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Candidate Gene Polymorphisms and Risk of Type-2 Diabetes Mellitus in Gaza Strip

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The Islamic University of Gaza

مكتب نائب الرئيس للبحث العلمي والدراسات العليا هاتف داخلي: ١١٥٠

الرقم: ج س غ/٣٥/ Ref:

التاريخ: ٢٠١٧/٠٤/١٦ Date:

نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ **اسراء عمر عبد الطلالقه** لنيل درجة الماجستير في **كلية *العلوم*** قسم **العلوم الحياتية- تحاليل طبية** وموضوعها:

التعدد الشكلي لجينات ذات صلة بخطر الإصابة بمرض السكري من النوع الثاني في قطاع غزة

Candidate Gene Polymorphisms and Risk of Type-2 Diabetes Mellitus in Gaza Strip

وبعد المناقشة التي تمت اليوم الأحد ٠٧ رجب ١٤٣٨هـ، الموافق ٢٠١٧/٠٤/١٦ الساعة الحادية عشرة صباحاً في قاعة مؤتمرات مبنى اللحيدان، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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أ.د. محمد عيد شبير	مناقشاً داخلياً	....
د. نبيل عبد الله العيلة	مناقشاً خارجياً	....

وبعد المداولة أوصت اللجنة بمنح الباحثة درجة الماجستير في **كلية *العلوم*/** قسم **العلوم الحياتية - تحاليل طبية**.

واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن يسخر علمها في خدمة دينها ووطنها.

والله ولي التوفيق ،،،

نائب الرئيس لشئون البحث العلمي والدراسات العليا

أ.د. عبدالرؤوف علي المناعمة

Abstract

Background: Type 2 Diabetes Mellitus (T2DM) is a multifactorial disease that results from the interaction between multiple genetic and environmental factors. Genome-wide association studies revealed many T2DM-associated genetic polymorphisms in various populations. Among the genes polymorphisms that were strongly associated with diabetes are (*PPARG rs1801282*), (*TCF7L2 rs7903146*), (*SLC30A8 rs13266634*), (*CDKAL1 rs10946398*), and (*KCNJ11 E23K*).

Objective: To investigate the association between (*PPARG rs1801282*), (*TCF7L2 rs7903146*), (*SLC30A8 rs13266634*), (*CDKAL1 rs10946398*), and (*KCNJ11 E23K*). genes polymorphisms and T2DM in the Gaza strip population.

Methods: In this case-control study 100 T2DM male patients and 100 control men were examined. The two groups were genotyped for the five genes polymorphisms using restriction fragment length polymorphism-PCR (RFLP-PCR) technique. Body mass index (BMI), glycated hemoglobin (HbA1c), insulin (C-peptide), total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were measured for all the study participants. The relation between the five genes polymorphisms, T2DM and the measured clinical parameters were statistically analyzed using appropriate tests.

Results: Among the tested polymorphisms, significant association was evident only between *KCNJ11 E23K* polymorphism and T2DM (P = 0.03). The *TCF7L2* "TT" genotype was significantly associated with increased HDL-C levels in both patients and controls (*P* values = 0.01 and 0.04, respectively). Moreover, the "TT" genotype proved to be significantly associated with lower plasma LDL-C in the patient group (P = 0.001). The *SLC30A8* "TT" genotype showed a significant effect on BMI in the control subjects (P value = 0.01) and on lower HbA1c level in the patient group (P value = 0.02). There was a significant increase in the means of BMI, triglycerides and HbA1c in patients as compared to controls (P values = 0.00, 0.02, and 0.00, respectively).

Conclusion: *KCNJ11* E23K is significantly associated with T2DM in the Gaza strip population. The *TCF7L2* "TT" genotype is significantly related to increased HDL-C level in the study population and to lower plasma LDL-C in the patients group. The *SLC30A8* "TT" genotype is significantly correlated with BMI in the control group. The means of BMI, triglycerides and HbA1c are significantly increased in patients as compared to controls. The study recommends confirming the obtained results on a larger sample, and examining the association of other genes polymorphisms with T2DM in Gaza strip.

Keywords: (*PPARG)*, (*TCF7L2)*, (*SLC30A8*), (*CDKAL1*),(*KCNJ11 E23K*), polymorphism ,type 2 diabetes mellitus, T2DM, Gaza strip.

الملخص

المقدمة: مرض السكري من النوع الثاني هو مرض متعدد العوامل وينشأ عن التفاعل بين عوامل وراثية وبيئية متعددة. كشفت دراسات العلاقة بين العوامل الوراثية على نطاق الجينوم ومرض السكري في مجموعات سكانية مختلفة عن وجود علاقة بين الانماط المختلفة لجينات محددة وخطر الاصابة بمرض السكري. وكان من بين الانماط الوراثية التي ارتبطت بشكل قوي مع حدوث مرض السكري تلك الموجودة في الجينات التالية: **(TCF7L2 rs7903146)**، **(*PPARG* rs1801282)** ، **(*SLC30A8* rs13266634)**، **(*CDKAL1* rs10946398)**، و.**(*KCNJ11* E23K)**.

الهدف: دراسة العلاقة بين خمس أنماط لخمس جينات مختلفة وهي :(*SLC30A8rs13266634*), (*PPARG rs1801282*) (*TCF7L2 rs7903146*), (*KCNJ11 E23K*),(*CDKAL1 rs10946398*) و خطر حدوث مرض السكري من النوع الثاني لدى الرجال في قطاع غزة.

الطرق المستخدمة: منهج الدراسة (مجموعة مرضية – مجموعة ضابطة) المجموعة المرضية تحتوي على100 رجل مريض بداء السكري من النوع الثاني, والمجموعة الضابطة تحتوي على100 رجل من الأصحاء. و قد تم إجراء المقابلة الشخصية لتعبئة الاستبيان, كما تم فحص الطراز الجيني للخمس جينات باستخدام تقنية (RFLP-PCR), بالإضافة الى قياس مخزون السكر, الأنسولين (c-ببتايد), الكولسترول, الدهون الثلاثية، الكولسترول عالي الكثافة, الكولسترول منخفض الكثافة. وتم تحليل البيانات والنتائج باستخدام الاختبارات الإحصائية المناسبة.

النتائج: أظهرت نتائج هذه الدراسة وجود علاقة ذات دلالة إحصائية (P = 0.03) بين (*KCNJ11 E23K*) وحدوث مرض السكري من النوع الثاني في قطاع غزة في حين لم تثبت وجود علاقة ذات دلالة إحصائية بين ,(*SLC30A8*),(*TCF7L2*) (*PPARG*), (*CDKAL1*) وحدوث مرض السكري من النوع الثاني. كما أشارت الدراسة إلى أن وجود الطراز الجيني "TT" لجين *TCF7L2* يزيد من مستوى البروتين الدهني عالي الكثافة عند كل من المرضى (P=0.01) والاصحاء (P=0.04), اضافة لذلك فان وجود هذا الطراز الجيني يعمل على خفض البروتين الدهني منخفض الكثافة عند المرضى. **(P =0.001)** و كما أظهرت الدراسة ان الطراز الجيني" TT " لجين *SLC30A8* كان له ارتباط ايجابي مع كل من كتلة الجسم (P=0.01) عند الاصحاء , وخفض مخزون السكر عند المرضى (P=0.02). وعرضت النتائج ايضا زيادة في متوسطات كل من: مخزون السكر, الدهون الثلاثية, و مؤشر كتلة الجسم عند المرضى بالمقارنة مع الاصحاء وكانت هذه النتيجة ذات دلالة إحصائية , (مؤشر كتلة الجسم (P=0.00), مخزون السكر(P=0.00), الدهون الثلاثية (P =0.02).

الخلاصة: نستنتج من هذا البحث وجود علاقة ذات دلالة إحصائية بين (*KCNJ11 E23K*) وحدوث مرض السكري من النوع الثاني في قطاع غزة. كما بينت الدراسة عن وجود علاقة ذات دلالة احصائية بين الطراز الجيني "TT" لجين *TCF7L2* و ارتفاع مستوى البروتين الدهني عالي الكثافة عند كل من المرضى والاصحاء وانخفاض البروتين الدهني منخفض الكثافة عند المرضى, اما الطراز الجيني" TT " لجين *SLC30A8* كان له ارتباط ايجابي مع كل كتلة الجسم عند الاصحاء , و انخفاض نسبة مخزون السكر عند المرضى. و لاحظنا ايضا زيادة في متوسطات كل من: مخزون السكر, الدهون الثلاثية, و مؤشر كتلة الجسم عند المرضى بالمقارنة مع الاصحاء وكانت هذه النتيجة ذات دلالة إحصائية.

وتوصي الدراسة: بإعادة فحص الجينات التي لم تعط دلاله احصائية مع زيادة حجم عينة الدراسة وخاصة لجين *TCF7L2*, وايضا بإجراء المزيد من الدراسات باستخدام جينات اخرى و تحديد علاقتها بمرض السكري من النوع الثاني في قطاع غزة.

كلمات افتتاحية: (*TCF7L2*), (*PPARG*), (*SLC30A8*), (*KCNJ11*),(*CDKAL1*)، التعدد الشكلي ، مرض السكري من النوع الثاني، قطاع غزة.

Dedication

I dedicate this modest work to my dearest parents, my **Father** who in spite of all odds, made every possible effort to make me finish my graduate study .

To my beloved **Mother** on whom the beauty of the universe lies.

To my sister **Doodie** who is the spirit of this life, her husband, my brother **Elian**, and their beautiful little kids **Omar** and **Sham**.

To my brothers: **Suhaib**, **Mohammad Abu Karam**, **Amourie**, **Aboud**, and ***Izzoo*** who lives abroad and striving to see us.

To my sister and my childhood friend ***Aya***.

Finally, firstly, always, and forever, I'd like to dedicate this with love and respect to the most beautiful person in my life, to all the love all the adoration my dearest friend uncle ***Husein***.

Israa Omar Altalalqa

Acknowledgements

I am grateful to **Allah**, who granted me life, power, peace and courage to finish this study.

Foremost, I would like to express my sincere gratitude to my advisor **Prof. Dr. Fadel A. Sharif** for the continuous support of my Master study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master study.

I want to express my deep thanks to the Faculty of Health Sciences and the Department of Biotechnology, and a special thanks to the **Dr. Mazen Alzaharna** for helping me in completing this study

Special thanks go to all members of the IUG Genetics lab and the Palestinian Medical Relief Society lab in particular **Mr. Mohammmed Ashour, Mr. Shadi El-Ashi**, **Mr. Mohamed alefash and Ms. Amani aljmmal,** that provided the support and equipment I have needed to produce and complete my thesis

I would like to express my thanks also to my friends, specially, my friend and sister **Mariam R. Al-Refi** for their help and support.

Finally, profuse thanks, love and appreciations to my **uncle** and my **friend Col. Husein Altalalqa** for all unlimited support and encouragement they provided me.

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List of Abbreviations

ADA	American Diabetes Association
BMI	body mass index
βGK	β-cells glucokinase
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosine Monophosphate
CDKAL1	Cdk5 Regulatory Subunit-Associated Protein 1-Like 1
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunoassay
FTO	Fat Mass And Obesity Associated
GDM	Gestational Diabetes Mellitus
GIP	Glucose-Dependent Insulinotropic Polypeptide
GIPR	Glucose-Dependent Insulinotropic Polypeptide Receptor
GL	Glycemic Load
GLP1R	Glucagon-Like Peptide-1 Receptor
GLUT2	Glucose Transporter Type 2
GLUT4	Glucose Transporter Type 4
GSIS	Glucose-Stimulated Insulin Secretion
GWAS	Genome-Wide Association Studies
HbA1c	Hemoglobin Glycated (Hemoglobina1c)
HDL-C	High Density Lipoprotein Cholesterol
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
K-ATP	ATP-Sensitive Potassium Channel
kb (= kbp)	Kilo Base Pairs
KCNJ11	Potassium Voltage-Gated Channel Subfamily J Member 11
LDL-C	Low Density Lipoprotein Cholesterol
MENA	Middle East And North Africa
mM	Millimolar
MODY	Maturity–Onset Diabetes Of The Young
NIDDM	Noninsulin-Dependent Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
***PPARG*(γ)**	Peroxisome Proliferator-Activated Receptor Gamma
PPRE	PPAR Response Element
RFLP	Restriction Fragment Length Polymorphism
SLC30A8	Solute Carrier Family 30, Member 8
SNP	Single Nucleotide Polymorphism
SUs	sulphonylureas
T1DM	Type I Diabetes Mellitus
T2DM	Type II Diabetes Mellitus

TCF7L2	Transcription Factor 7-Like 2
TG	Triglycerides
TZDs	Thiazolidinediones
VDR	Vitamin D Receptor
WHO	World Health Organization
ZNT	Zinc Transporter

Chapter 1

Introduction

Chapter 1
Introduction

1.1 Overview

Diabetes mellitus (DM) is a common health problem worldwide, it is one of the world's most important causes of healthcare expenditure, mortality, morbidity and lost economic growth.

DM is defined as a metabolic disorder characterized by chronic hyperglycemia due to disturbances of carbohydrate, fat and protein metabolism that are associated with absolute or relative deficiencies in insulin secretion, insulin action or both (**American Diabetes Association, ADA, 2014**). There are two major types of DM: Type 1 diabetes which is primarily a result of pancreatic β-cell destruction due to an immune-mediated process that is likely incited by environmental factors in genetically predisposed individuals (**Harjutsalo, Reunanen, & Tuomilehto, 2006**). The more prevalent form, type 2 diabetes, usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce it (**Cohen, 2006**).

The International Diabetes Federation has estimated that the number of people with diabetes is expected to rise from 366 million in 2011 to 552 million by 2030 if no urgent action is taken. Furthermore, many people are unaware that they have diabetes, with a number around 183 million. Type 2 diabetes mellitus (T2DM) represents > 90% of the cases (**Lyssenko, & Laakso, 2013**). In Palestine, as in other countries, T2DM seems to be a serious health problem among the population with a prevalence rate of around 9% - 12% (**Husseini et al., 2009**).

T2DM is a complex multifactorial disease in which multiple genetic variants interact with environmental factors to trigger the disease (**Lyssenko, 2008**). There is sufficient evidence that T2DM has a strong genetic basis. The concordance of T2DM in monozygotic twins is ~76% (**Medici, Hawa, Ianari, Pyke & Leslie, 1999**). The lifetime risk (at age 80 years) for T2DM has been calculated to be 38% if one parent had T2DM. If both parents are affected, the incidence of T2DM in the offspring is estimated to approach 60% by the age of 60 years (**Stumvoll, Goldstein & van**

Haeften, 2005). Advances in genotyping technology have facilitated rapid progress in large-scale genetic studies. Since 2007, genome-wide association studies (GWAS) have identified >65 genetic variants that increase the risk of T2DM by 10–30% **(Morris et al., 2012)**. Recent technological developments have allowed the successful identification of common single nucleotide polymorphisms (SNPs) contributing to diabetes susceptibility **(Takeuchi et al., 2009)**. So far, 10 SNPs have been reported in multiple studies and meta-analyses as significantly associated with increased risk of T2DM.

In Gaza strip, studies on SNPs in different proposed risk genes and increased risk of T2DM are limited and restricted to Calpain-10 gene **(El Zaharna, 2005)** and *KCNJ11* gene **(Abed, Ayesh, Hamdona, 2013)**. However, no previous studies examined SNPs in other documented risk genes (e.g., Peroxisome proliferator-activated receptor gamma (*PPARG*); transcription factor 7-like 2 (*TCF7L2*); solute carrier family 30, member 8 (*SLC30A8*); CDK5 Regulatory Subunit-Associated Protein 1-Like 1 (*CDKAL1*).

1.2 Significance

In Gaza strip, no previous studies investigated the relation between SNPs in reported risk genes (*PPARG)*, (*TCF7L2*), (*SLC30A8*) and (*CDKAL1*) and the risk of T2DM. Identifying individuals at risk of developing T2DM, through investigating risk alleles, is important both for investigators and health care providers to take the appropriate measures for delaying disease, prevention of disease onset and/or its associated complications and for selecting the appropriate treatment.

1.3 General objective

The overall aim of this work is to investigate the relation between SNP in five candidate genes and risk of having T2DM in Gaza Strip.

1.4 Specific objectives

1. To investigate the relation between documented SNPs in *PPARG, TCF7L2*, *KCNJ11*, *CDKAL1* and *SLC30A8* and the risk of having T2DM.
2. To determine the genotypic and allelic frequencies of 5 SNPs at PPARG, *TCF7L2*, *KCNJ11*, *CDKAL1* and *SLC30A8* in the study population.

3. To evaluate the glycemic status of T2DM patients and healthy controls using whole blood hemoglobin A1c (HbA_{1C}) and insulin (C-peptide).
4. To compare T2DM patients and healthy controls in terms of their BMI and lipid profile (total cholesterol, triglycerides, high density lipoprotein cholesterol "HDL-C" and low density lipoprotein cholesterol "LDL-C").
5. To investigate the relation between the five SNPs and BMI, HbA1c, C-peptide, and the lipid profile (total cholesterol, triglycerides, HDL-C, and LDL-C) in the T2DM patients and the control subjects.

Chapter 2

Literature Review

Chapter 2
Literature Review

2.1 Diabetes mellitus

2.1.1 Definition of diabetes mellitus and pre-diabetes

Diabetes mellitus is a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both. It is defined as fasting blood glucose concentration above 126.126 mg/dL (7 mM), or above 198.198 mg/dL (11 mM) two hours after ingestion of 75 grams of glucose. The chronic hyperglycemia of diabetes is associated with relatively specific long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD). Pre-diabetes is a practical and convenient term referring to impaired fasting glucose (IFG), a person has a blood glucose level between 100 and 125 mg/dl, impaired glucose tolerance (IGT), the 2-hour blood glucose is between 140 and 199 mg/dl or a glycated hemoglobin (HbA1c) of 6.0% to 6.4%, each of which places individuals at high risk of developing diabetes and its complications **(American Diabetes Association, ADA, 2014)**.

2.2 Types of diabetes

2.2.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is characterized by loss of the insulin-producing β-cells of the islets of Langerhans in the pancreas, leading to insulin deficiency. This type can be further classified as immune-mediated or idiopathic. The majority of T1DM is of the immune-mediated nature, in which a T-cell-mediated autoimmune attack leads to the loss of β-cells and thus insulin **(Rother, 2007)**. It causes approximately 10% of diabetes mellitus cases in North America and Europe. Most affected people are otherwise healthy and of a healthy weight when onset occurs. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages. T1DM can affect children or adults, but was traditionally termed "juvenile diabetes" because a majority of these diabetes cases were in children **(Dunger, & Todd, 2008)**.

2.2.2 Type 2 Diabetes Mellitus

Type 2 Diabetes mellitus (T2DM) ranges from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance. This form of diabetes, which accounts for 90–95% of those with diabetes, previously was referred to as non–insulin dependent diabetes, or adult-onset diabetes **(Genuth et al., 2013)**.

2.2.3 Gestational diabetes

The American Diabetes Association defines gestational diabetes mellitus (GDM) as carbohydrate intolerance of variable severity, with onset or first diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation **(American Diabetes Association, ADA, 2017)**.

2.2.4 Other rare types of DM

Several forms of the diabetic state may be associated with single gene (monogenic) defects in β-cells function. Maturity-onset diabetes of the young (MODY) is a group of monogenic disorders characterized by autosomal dominantly inherited non-insulin dependent form of diabetes classically presenting in adolescence or young adults before the age of 25 years. MODY is a rare cause of diabetes (1% of all cases) and is frequently misdiagnosed as T1DM or T2DM. People with MODY are generally not overweight and do not have other risk factors for T2DM, such as high blood pressure or abnormal blood fat levels **(Anık, Çatlı, Abacı & Böber, 2015)**.

2.3 Prevalence and mortality rate of diabetes mellitus

2.3.1 Mortality rate

Worldwide, one and a half million people died directly from diabetes in 2014. However, blood glucose levels that are higher than optimal but are below the diagnostic threshold for diabetes also increases the risk of death, particularly from CVDs, and have caused an additional 2.2 million deaths. The largest number of deaths due to high blood glucose has occurred in upper middle-income countries. Almost one-half of all deaths attributable to high blood glucose are premature - before the age of 70 years. In low-income countries, more than half of the deaths

attributable to high blood glucose are premature, in contrast to high-income countries where about one-fifth of deaths are premature. This is probably the result of later detection and poorer management in low-income countries. High blood glucose age-standardized mortality rates per 100 000 people 20-year-old and older range from 55.7 in the WHO European region to 138.3 in the WHO Eastern Mediterranean region (**Roglic, 2016**). WHO projects that diabetes will be the 7th leading cause of death in 2030 (**Mathers & Loncar, 2006**).

2.3.2 Prevalence and incidence

The number of people with diabetes has increased from 180 million in 1980 to 422 million in 2014. This increase is attributed to population growth and aging (40%), rise in age-specific prevalence (28%) and interaction of the two (32%). Age-standardized prevalence trends are shown in Figure (2.1). Age-standardized prevalence in adults 18-year-old and above has almost doubled, from 4.7% in 1980 to 8.5% in 2014. In 1980, the prevalence was highest in high-income countries (5.2%) and lowest in low-income countries (3.3%). By 2014, the prevalence in low-income countries has become higher (7.4%) than in high-income (7.0%) countries. The prevalence of diabetes has risen faster in low- and middle-income countries and is currently highest in upper middle-income countries (9.3%). In the WHO regions, the prevalence is highest in the Eastern Mediterranean region (13.7%) and lowest in the African region (7.1%)(**IDF Diabetes Atlas, 2015**).

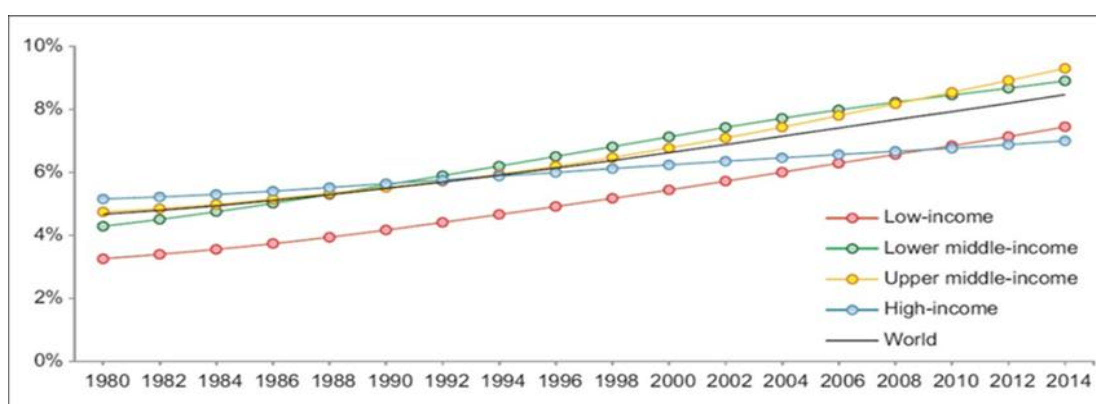

Figure (2.1): Trends in prevalence of diabetes, 1980-2014, by country income group (IDF Diabetes Atlas, 2015).

In 2015, the top 10 countries with higher number of people with diabetes are shown in Table (2.1) **(IDF Diabetes Atlas, 2015).**

Table (2.1): Top ten countries/territories for number of people with diabetes (20-79 years), 2015.

#	Country/territory	Number of people with diabetes	Diabetes prevalence
1	China	109.6 million	0.008%
2	India	69.2 million	0.005%
3	United States of America	29.3 million	9.18%
4	Brazil	14.3 million	4.13%
5	Russian Federation	12.1 million	8.43%
6	Mexico	11.5 million	9.40%
7	Indonesia	10.0 million	4.00%
8	Egypt	7.8 million	9.50%
9	Japan	7.2 million	5.65%
10	Bangladesh	7.1 million	4.38%

2.3.3 Prevalence and incidence in Palestine

Palestine is one of the 19 countries and territories of the IDF Middle East and North Africa (MENA) region. Worldwide, 415 million people have diabetes and more than 35.4 million people in the MENA Region; by 2040 this will rise to 72.1 million. There are over 146,700 cases of diabetes in Palestine with a prevalence in adults (20-79 years) of 6.5%. On the other hand, the number of cases of diabetes in adults that are undiagnosed is 59,500 cases in adult (20-79 years) Palestinian population. Many middle- and low-income countries (as Palestine) have more people under the age of 60 with diabetes compared to the world average. Meanwhile, for high-income countries, a growing population over the age of 60 makes up the largest proportion of diabetes prevalence **(IDF Diabetes Atlas, 2015).**

2.4 Risk factors for T2DM

Many studies have elaborated the associations between several risk factors and occurrence of T2DM. Body mass index (BMI), lipids, smoking, physical inactivity, low education, dietary patterns, vitamin D deficiency, family history, and recently

specific genes polymorphisms are the most frequently documented risk factors for developing T2DM **(Valdes, Botas, Delgado, Alvarez & Cadorniga, 2007; Lyssenko et al., 2008)**.

2.4.1 BMI

Overweight and obesity are driving the global diabetes epidemic. They affect the majority of adults in most developed countries and are increasing rapidly in developing countries. If current worldwide trends continue, the number of overweight people (BMI ≥25 kg/m2) is projected to increase from 1.3 billion in 2005 to nearly 2.0 billion by 2030 **(Mathers & Loncar, 2006)**.

Many longitudinal studies have reported that increased BMI is a strong risk factor for T2DM **(Meisinger, Thorand, Schneider, Stieber & Doring, 2002; Almdal, Scharling, Jensen & Vestergaard, 2008)**. A strong positive association between obesity and T2DM is found both in men and women **(Skarfors, Selinus & Lithell, 1991)**. Obesity is associated with increased risk of developing insulin resistance and T2DM. In obese individuals adipose tissue releases increased amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other factors involved in the development of insulin resistance. When insulin resistance is accompanied by dysfunction of the β-cells, the following fall in insulin secretion results in failure to control blood glucose level leading to T2DM.

Many genes interact with the environment leading to obesity and in some also to diabetes. Many genes have been shown to be involved in determining the whole range of BMI in a population, with each gene only explaining a few hundred grams difference in body weight **(Hebebrand & Hinney, 2009)**. Genes responsible for obesity and insulin resistance interact with environmental factors such as increased fat/ calorie intake and decreased physical activity resulting in the development of obesity and insulin resistance followed ultimately by the development of T2DM **(Kahn, Utzschneider, & Hull, 2006).**

2.4.2 Lipids

Unfavorable blood lipids has been reported as a risk factor for T2DM by several prospective studies. An inverse relationship between HDL cholesterol and

risk of T2DM have been documented **(Jacobsen, Bonaa, & Njolstad, 2002)**. High plasma triglycerides and low plasma HDL cholesterol levels are both seen in the insulin resistance syndrome, which is a pre-diabetic state **(Taskinen, 2003)**.

The mechanisms suggested are increased circulating levels of free fatty acids due to increased insulin levels and increased chylomicron-assembly and secretion in the gut, the latter process being a result of localized insulin resistance in the intestine. Cross sectional studies have shown that high BMI is associated with a higher level of total cholesterol and unfavorable lipids pattern, with low concentrations of HDL cholesterol and high triglycerides concentrations **(Tsai , Yang, Lin & Fang, 2004; Wild & Byrne, 2006)**.

2.4.3 Smoking

Several prospective studies reported that current smoking is a risk factor for developing T2DM **(Yeh , Duncan, Schmidt, Wang, & Brancati, 2010)**. The association between smoking and T2DM was stronger for heavy smokers $\geq$ 20 cigarettes/day compared with light smokers or former smokers **(Nagaya , Yoshida, Takahashi, & Kawai, 2008)**. In addition some studies found an increased risk of T2DM the first 2-3 years after smoking cessation **(Hur et al., 2007)**. Smoking leads to insulin resistance and inadequate compensatory insulin secretion response. This could be due to a direct effect of nicotine or other components of cigarette smoke on β-cells of the pancreas as suggested by the association of cigarette smoking with chronic pancreatitis and pancreatic cancer **(Talamini et al., 1999)**.

2.4.4 Physical inactivity

Strong evidence in many studies shows that physical inactivity increases the risk of many adverse health conditions, including major non-communicable diseases such as T2DM. Longitudinal studies have found physical inactivity to be a strong risk factor for T2DM **(Fretts et al., 2009)**. Prolonged television watching as a surrogate marker of sedentary lifestyle, was reported to be positively associated with diabetes risk in both men and women. Moderate and vigorous physical activity was associated with a lower risk of T2DM **(Krishnan , Rosenberg, & Palmer, 2009)**. Physical activity plays an important role in delaying or prevention of development of T2DM in those at risk both directly by improving insulin sensitivity

and reducing insulin resistance, and indirectly by beneficial changes in body mass and body composition (**Hamman et al., 2006**).

2.4.5 Dietary pattern

Excessive caloric intake is a major driving force behind escalating obesity and T2DM epidemics worldwide. Dietary habits are important life style factor associated with the development of T2DM. Positive association have been reported between the risk of T2DM and different patterns of food intake. Many studies found that the quality of fats and carbohydrates plays an important role in the development of diabetes, independent of BMI and other risk factors **(Hu et al., 2001).** Higher dietary glycemic load (GL) and trans fat are associated with increased diabetes risk, whereas greater consumption of cereal fiber and polyunsaturated fat is associated with decreased risk **(de Munter, Hu, Spiegelman, Franz, & van Dam, 2007**). Higher consumption of fruits and vegetables is associated with reduced risk of T2DM. The possible mechanisms suggested are that insoluble fiber intake was consistently associated with improved insulin sensitivity **(Meyer et al., 2000)**. A prospective study found that regular consumption of white rice is associated with an increased risk of T2DM whereas replacement of white rice by brown rice or other whole grains is associated with a lower risk **(Sun et al., 2010)**.

2.4.6 Vitamin D deficiency

A recent research showed that vitamin D deficiency may have negative effects on glucose intolerance, insulin secretion and T2DM , either directly via vitamin D receptor (VDR) activation or indirectly via calcemic hormones and also via inflammation **(Chagas, Borges, Martini, & Rogero, 2012**). As both 1-α-hydroxylase and VDR are present in pancreatic β-cells, vitamin D has significant roles in the synthesis and release of insulin. Furthermore, vitamin D has influence on the insulin sensitivity by controlling calcium flux through the membrane in both β-cells and peripheral insulin-target tissues (**Wolden-Kirk, Overbergh, Christesen, Brusgaard, & Mathieu, 2011**). In addition, vitamin D supplementation is recognized as a promising and inexpensive therapy, which may decrease the risk of T2DM and improve glycemic parameters in T2DM patients. Therefore, it seems that the positive effects of vitamin D are correlated with its action on insulin secretion

and sensitivity as well as on inflammation (**Takiishi, Gysemans, Bouillon, & Mathieu, 2010**).

2.4.7 Genetics

There is sufficient evidence that T2DM has a strong genetic basis (**Medici et al., 1999**). The concordance of T2DM in monozygotic twins is ~76%. The lifetime risk (at age 80 years) for T2DM has been calculated to be 38% if one parent had T2DM. If both parents are affected, the incidence of T2DM in the offspring is estimated to approach 60% by the age of 60 years (**Stumvoll, Goldstein, & Haeften, 2008**).

Data from multiple laboratories support the notion that genetic factors predispose to development of T2DM by reducing insulin sensitivity and insulin secretion, which deteriorate in parallel in most human T2DM cases (**Das , & Elbein, 2006**). Several studies have identified variants in 11 genes (*TCF7L2*, *PPARG*, *FTO*, *KCNJ11*, *NOTCH2*, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1*, and *HHEX*) to be significantly associated with the risk of T2DM independently of other clinical risk factors and variants in 8 of these genes were associated with impaired β-cells function. Among these genes expressed in pancreatic cells and involved in impairment of insulin secretion, the transcription factor 7-like 2 (TCF7L2), is the locus with the highest risk for T2DM (**Lyssenko et al., 2008; Prokopenko, McCarthy & Lindgren, 2008**).

2.5 Genetic susceptibility to T2DM

Technological developments in molecular biology have allowed the successful identification of common single nucleotide polymorphisms (SNPs) contributing to diabetes susceptibility. Genome-wide approaches, such as genome-wide association studies (GWAS), have been successful at finding statistically significant associations between specific genomic loci and T2DM susceptibility (**Basile et al., 2014; Al Safar et al., 2013**).

2.5.1 Genetic variants associated with T2DM

As of the beginning of 2014, 90 genetic loci have been firmly established as T2DM risk loci (**Albrechtsen, Grarup, & Li, 2013; Steinthorsdottir,**

Thorleifsson & Sulem, 2014). There are, however, significant interethnic differences in the location and frequency of these risk alleles **(Hu, 2011)**. The risk variant in the *TCF7L2* locus, which was discovered in 2006 by a positional linkage strategy in the Icelandic population remains the most influential common T2DM variant **(Grant, Thorleifsson, & Reynisdottir, 2006)**.

Table 4.1 illustrates the genetic loci that have been firmly established as T2DM risk alleles along with discovery method, major ethnicity, and cellular function, and putative intermediary mechanism(s) in diabetes.

Table (2.1): Genetic regions (variants) associated with T2DM at genome-wide levels of statistical significance ($p < 10^{-6}$), listed by chromosome.

Chr.	Gene region (lead SNP)	Discovery method (major ethnicity)	Cellular function and putative intermediary mechanism in diabetes
1	*PROX1 (rs340874)*	Follow-up of signals GWA scan for FG (European)	Encodes the prospero-related homeobox 1. Implicated in cell proliferation and development. Associated with elevated FG.
1	*NOTCH2 (rs10923931)*	GWA meta-analysis (European)	Transmembrane receptor implicated in pancreatic organogenesis; regulates cell differentiation.
2	*GRB14 (rs3923113)*	GWA meta-analysis (South Asians)	Adaptor protein binding to insulin receptor and insulin-like growth factor receptors. Associated with reduced insulin sensitivity.
2	*BCL11A (rs243021)*	GWA meta-analysis (European)	Involved in both B- and T-lymphocyte development and β-cell function. Affects insulin response to glucose.
2	*RBMS1 (rs7593730)*	GWA meta-analysis (European)	Encodes RNA-binding motif, single-stranded interacting protein. Implicated in DNA replication, gene transcription, cell cycle progression and apoptosis. Unknown diabetogenic mechanism.
2	*GCKR (rs780094)*	Single GWA study, scan for FG (European)	Glucokinase regulatory protein. Involved in signal transduction, glucose transport and sensing. Associated with FG, fasting insulin and HOMA-IR.
2	*IRS1 (rs2943641)*	Single GWA study (French, European)	Encodes insulin receptor substrate-1. Associates with reduced adiposity and impaired metabolic profile (e.g. visceral to subcutaneous fat ratio, IR, dyslipidemia, CVD, adiponectin levels).
2	*THADA (rs7578597)*	GWA meta-analysis (European)	Thyroid adenoma-associated gene. Associates with PPAR; Involved in apoptosis. Associated with β-cell dysfunction, lower β- cell response to GLP-1 and reduced β-cell mass.
3	*ST6GAL1 (rs16861329)*	GWA meta-analysis (South Asians)	Enzyme located in Golgi apparatus, involved in post-translational modification of cell-surface components by glycosylation.
3	*ADCY5 (rs11708067)*	Single GWA study, scan for FG	Encodes adenylate cyclase 5. Involved in signal transduction. Associated with elevated FG.

		(European)	
3	*ADAMTS9 (rs4607103)*	GWA meta-analysis (European)	Proteolytic enzyme. Affects insulin response to glucose. Primary effect on insulin action not driven by obesity
3	*IGF2BP2* (rs4402960)	Single GWA study (European)	Growth factor (IGF2-mRNA) binding protein. Involved in pancreatic development and stimulation of insulin action.
3	*PPARG* (rs1801282)*	Candidate study; Later confirmation by GWA studies	TRF involved i adipocyte development. TRF receptor for TZDs and prostaglandins. Effect on IR.
4	*WFS1* (rs1801214)*	Candidate study; later validated by GWA meta-analysis	Endoplasmic reticulum transmembrane protein involved in stress and β-cell apoptosis. Insulin response.
5	*ZBED3 (rs4457053)*	GWA meta-analysis (European)	Encodes an axin-interacting protein activating wnt/beta-catenin signaling. Unknown diabetogenic mechanism.
6	*GLP1R (rs10305492)*	Follow-up of signals for T2D from GWA scan for FG (European)	GLP-1 has a range of downstream actions including(glucose-dependent stimulation of insulin release, inhibition of glucagon secretion from the islet alpha-cells, appetite suppression). Minor A allele was associated with lowering FG
6	*ENPP1 K121Q*	Single GWA study (British, European)	The encoded protein is a type II transmembrane glycoprotein comprising two identical disulfide-bonded subunits, contribute to insulin resistance
6	*CDKAL1 (rs7754840)*	Single GWA study (Icelandic, European)	Cyklin kinase (CDK5) inhibitor. Involved in cell cycle regulation in the β-cell. Insulin response.
7	*KLF14 (rs972283)*	GWA meta-analysis (European)	Basic transcription element-binding protein. "Master switch" controlling other genes associated with BMI, insulin, glucose and cholesterol.
7	*DGKB (rs972283)*	Follow-up of signals for T2D from GWA scan for FG (European)	Encodes diacylglycerol kinase beta. Implicated in signal transduction. Associated with elevated FG.
7	*GCK* (rs4607517)*	Follow-up of signals for T2D from GWA scan for FG (European)	Encodes the enzyme glucokinase. Involved in signal transduction, glucose transport and sensing. Associated with elevated FG and HbA1c.
7	*JAZF1 (rs864745)*	GWA meta-analysis (European)	Zinc-finger protein. Function as a transcriptional repressor. Associated with prostate cancer. Insulin response.
8	*TP53INP1*	GWA meta-analysis (European)	(rs896854) Encodes the p53-dependent damage-inducible nuclear protein. May regulate p53-dependent apoptosis. Unknown diabetogenic mechanism.
8	*SLC30A8 (rs13266634)*	Single GWA study (French, European)	β-cell zinc transporter ZnT8. Involved in insulin storage and secretion. Associated with fasting proinsulin levels.

9	*TLE4a (rs13292136)*	GWA meta-analysis (European)	Encodes the transducin-like enhancer of split 4. Unknown diabetogenic mechanism.
9	*PTPRD (rs17584499)*	Single GWA study (Taiwanese)	Encodes the tyrosine phosphatase receptor type D protein. Associated with increased HOMA-IR and may affect insulin signaling on its target cells.
10	*VPS26A (rs1802295)*	GWA meta-analysis (South Asians)	Multimeric protein involved in transport of proteins from endosomes to the trans-Golgi network. Expressed in pancreatic and adipose tissues.
10	*CDC123 (rs12779790)*	GWA meta-analysis (European)	Cell cycle kinase, required for S-phase entry. Affects different aspects of insulin response to glucose.
10	*HHEX (rs1111875)*	Single GWA study (French, European)	TRF involved i pancreatic development. Might influence both insulin release and insulin sensitivity.
10	*TCF7L2 (rs7903146)*	Linkage study; later confirmation by several GWAs (European)	TRF involved in wnt-signaling. Influencing insulin and glucagon secretion. Most important polygene identified for T2D.
10	*TCF7L2 (rs12255372)*	Single GWA study (South African)	TRF involved in wnt-signaling. Influencing insulin and glucagon secretion. Most important polygene identified for T2D.
10	*TCF7L2 (rs4506565)*	Single GWA study (Asians)	TRF involved in wnt-signaling. Influencing insulin and glucagon secretion. Most important polygene identified for T2D.
11	*ARAP1b (rs1552224)*	GWA meta-analysis (European)	Associated with lower proinsulin levels, as well as lower β-cell function (HOMA-B and insulinogenic index).
11	*HMGA2 (rs1531343)*	GWA meta-analysis (European)	Oncogene implicated in body size (height). Primary effect on insulin action not driven by obesity.
11	*MTNR1B (rs10830963)*	Follow-up of signals for T2D from GWA scan for FG or IS	Receptor for melatonin. Involved in glucose homeostasis. Associated with increased FG and reduced β-cell function.
11	*KCNQ1 (rs2237892)*	Single GWA study (Japanese, Asian, European)	Encodes the pore-forming alfa subunit of IKaK+ channel. Insulin response.
11	*KCNJ11* (rs5219)*	Candidate study; later confirmation by GWAS	Inwardly rectifying potassium channel. Risk allele impairs insulin secretion.
12	*HNF1A* (rs7957197)*	Candidate study; GWA meta-analysis (European)	TRF essential for pancreatic β-cell development and function
12	*TSPAN8 (rs7961581)*	GWA meta-analysis (European)	Cell surface glycoprotein implicated in GI cancers. Insulin response.
13	*SPRY2 (rs1359730)*	Single GWA study (Chinese)	Inhibitor of tyrosine kinase signaling. Associated with body fat percentage. Homologs inhibit insulin receptor-transduced MAPK signaling. Regulates development of pancreas.

15	*AP3S2 (rs2028299)*	GWA meta-analysis (South Asians)	Clathrin-associated adaptor complex expressed in adipocytes and pancreatic islets. Involved in vesicle transport and sorting. Unknown diabetogenic mechanism
15	*HMG20A (rs7178572)*	GWA meta-analysis (South Asians)	High mobility group non-histone chromosomal protein influencing histone methylation. Involved in neuronal development. Unknown diabetogenic mechanism.
15	*C2CD4A (rs11071657)*	Single GWA study (Japanese)	Nuclear calcium-dependent domain-containing protein. Impairs glucose-stimulated insulin response. Associated with levels of fasting glucose and proinsulin.
15	*ZFAND6 (rs11634397)*	GWA meta-analysis (European)	Encodes a zinc finger AN1 Domain-containing protein. Unknown diabetogenic mechanism.
15	*PRC1 (rs8042680)*	GWA meta-analysis (European)	Protein regulating cytokinesis. Unknown diabetogenic mechanism.
16	*FTO (rs8050136)*	Single GWA study (British, European)	rs9939609) 2-oxoglutarate-dependent demethylase. Alters BMI i general population.
17	*SRR (rs391300)*	Single GWA study (Taiwanese)	Encodes a serine racemase protein. May play a role in regulation of insulin and glucagon secretion.
17	*HNF1B* (rs757210)*	Candidate study	TRF involved in development of the kidney, pancreas, liver, and Mullerian duct. Implicated in MODY and renal cyst. Associated with prostate cancer.
20	*HNF4A* (rs4812829)*	Candidate study; Later replicated by GWA meta-analysis (South Asians)	Nuclear TRF expressed in liver. Regulates transcription of several genes, e.g. HNF1A. Elevated hepatic glucose production. Defective pancreatic β-cell function and impaired insulin secretion.
X	*DUSP9 (rs5945326)*	GWA meta-analysis (European)	MAP kinase phosphatase. Decreased insulin release for male risk allele carriers. Up-regulated during adipocyte differentiation. Involved in insulin signaling and stress induced IR.

For many of the loci several SNPs associate with T2D, but only one (in some cases two) SNP is listed. Abbreviations: Chr: chromosome; CVD: cardiovascular disease; FG: fasting glucose; GI: gastrointestinal; IR: insulin resistance; IS: insulin secretion; TRF: transcription factor; TZD: thiazolidinediones. *Genes also implicated in MODY, other monogenic forms of diabetes or rare genetic syndromes. The table is compiled from (**Doria, Patti, & Kahn, 2008; Billings & Florez, 2010; Pettersen, Skorpen, Kvaløy, Midthjell, & Grill, 2010; Lango Allen et al., 2010; Garber, 2011; Molven & Njølstad, 2011; Yako et al., 2015; Acharya et al., 2015**).

2.6 Candidate genes

2.6.1 Peroxisome proliferator-activated receptor gamma (*PPARG rs1801282*)

2.6.1.1 Location of gene

The *PPARG* gene is about 100 kb long and comprises 9 exons. *PPARG1* is encoded by 8 exons and *PPARG2* by 7 exons. *PPARG1* uses exons A1 and A2, whereas *PPARG2* uses exon B; both use exons 1 through 6. The gene is located on the short (p) arm of chromosome 3 at position 25.2 (3p25.2) (**Fajas et al., 1997**).

2.6.1.2 Gene Function

Peroxisome proliferator-activated receptor *(PPAR)-γ* is a nuclear hormone receptor that comprises an agonist-dependent activation domain (AF-2), DNA binding domain, and agonist-independent activation domain (AF-1). It is expressed predominantly in adipose tissue but is expressed in other tissues as well. Upon the binding of the agonists, *PPAR-γ* heterodimerizes with retinoid X receptor-α and activates the transcription of target genes through the binding of the PPAR response element (PPRE) (**Auboeuf et al., 1997**).

2.6.1.3 Role in T2DM

Moderate amounts of *PPAR-γ* are expressed in pancreatic β-cells, and its expression is increased in the diabetic state. Thiazolidinediones (TZDs) are known to enhance pancreatic growth. But the fundamental role of PPAR-γ in β-cells is not fully understood. Currently, the reports on the effects of *PPAR-γ* on insulin secretion are contradictory. *PPAR-γ* agonists can decrease insulin secretion in diabetic animal models, whereas activation of *PPAR-γ* does not acutely improve insulin secretion in isolated human islets (**Jia & Otsuki, 2002; Harmon et al., 2000**). However, it is reported that PPAR-γ agonists can protect the β-cells from apoptosis and restore the function of β-cells, including glucose-stimulated insulin secretion (GSIS). Activation of PPAR-γ leads to restoration of the glucose-sensing ability of β-cells through the activation of glucose transporter isotype 2 (GLUT2) and pancreatic β-cells glucokinase (βGK) gene expression in diabetic subjects. The functional response element for *PPAR-γ* was identified in the promoters of GLUT2 and βGK (**Kim et al., 2000, 2002**). PPARγ is a regulator of lipid and glucose metabolism and therefore

its synthetic ligands such as thiazolidinediones improve insulin and glucose parameters and increase whole body insulin sensitivity. Therefore, they are called insulin-sensitizing medications and they are used in the treatment of diabetes **(Elstner et al., 1998)**.

In Kashmir, a study was conducted to investigate the possible role of *PPARγ2* Pro12Ala (*rs1801282*) polymorphism, as a genetic risk factor for T2DM. The study was performed on 200 Kashmiri population (100 T2DM patients and 100 controls). PCR–RFLP technique was used for genotyping analysis. The frequency of the Pro allele was 79 and 91.5 % for controls and cases, respectively ($P < 0.05$; OR 3.2; 95 % CI 1.64–6.3). The study found a significant association of Pro12Ala polymorphism of *PPARγ2* gene with T2DM. However, the genotypes showed statistically significant association only with few clinical parameters including BMI, total cholesterol, and low-density lipoprotein ($P < 0.05$). The study signifies that Pro allele in *PPARγ2* may be a genotypic risk factor that confers susceptibility to T2DM in the ethnic Kashmiri population **(Majid, Masood, Kadla, Hameed, & Ganai, 2016)**.

In another study **in Malaysia**, the possible role of *PPARG* (Pro12Ala) gene polymorphism as a genetic risk factor for T2DM patients was investigated. A total of 241 subjects between the age of 35 and 85 years were recruited for the study. Out of the total 241 subjects, 120 were T2DM patients and 121 were healthy individuals. *PPARG* (Pro12Ala) genotypes were determined by PCR-RFLP. The frequencies of wild homozygote (WH), heterozygote (H), and mutant homozygote (MH) among the T2DM patients were ($n = 73$) 60.8 %, ($n = 39$) 32.5 %, and ($n = 3$) 2.5 % as compared to ($n = 57$) 47 %, ($n = 46$) 38 %, and ($n = 16$) 13.2 % among the healthy subjects. The mean of HbA1c (%) among normal and diabetic patients with genotypes were different (5.36 ± 0.54 vs 7.58 ± 1.76), $p < 0.005$. The authors concluded that the *PPARG* (Pro12Ala) SNP could be a genetic risk factor for insulin resistance and T2DM among Malaysian subjects **(Paramasivam, Safi, Qvist, Abidin, Hairi, & Chinna, 2016)**.

2.6.2 Transcription factor 7-like 2 (*TCF7L2 rs7903146*)

2.6.2.1 Location of gene

The *TCF7L2* gene is located on the long (q) arm of human chromosome 10 at position q25.2-25.3 (10q25.2-q25.3) **(Yi, Brubaker, & Jin , 2005)**.

2.6.2.2 Gene Function

TCF7L2 participates in the Wnt signaling pathway (Wnt family of secreted glycolipoproteins, via the transcription co-activator β-catenin) controls embryonic development and adult homeostasis and modulates *MYC* (a regulator gene that codes for a transcription factor) expression by binding to its promoter in a sequence-specific manner. TCF7L2 acts as a repressor in the absence of *CTNNB1* (the protein encoded by this gene is part of a complex of proteins that constitute adherens junctions), and as an activator in its presence. It activates transcription from promoters with several copies of the Tcf motif 5'-CCTTTGATC-3' in the presence of *CTNNB1*. TLE1, TLE2, TLE3 and TLE4 (involved in the control of haemopoiesis, neuronal differentiation and terminal epithelial differentiation) repress transactivation mediated by *TCF7L2/TCF4* and *CTNNB1*. Expression of dominant-negative mutants results in cell-cycle arrest in G1. TCF7L2 is also necessary for the maintenance of the epithelial stem-cell compartment of the small intestine **(He et al., 1998)**.

2.6.2.3 Role in T2DM

Decreased TCF7L2 protein levels in pancreatic sections from 7 patients with T2DM (Noninsulin-Dependent Diabetes Mellitus, NIDDM; 125853) compared with 7 healthy controls has been reported. Expression of the receptors for glucagon-like peptide-1 (GLP1R) and glucose-dependent insulinotropic polypeptide (GIP) was decreased in human T2DM islets as well as in isolated human islets treated with siRNA to *TCF7L2* (*siTCF7L2*). Insulin secretion stimulated by glucose, GLP1, and GIP, but not KCl or cyclic adenosine monophosphate (cAMP), was impaired in siTCF7L2-treated isolated human islets. Loss of *TCF7L2* resulted in decreased GLP1 and GIP-stimulated AKT (AKT1) phosphorylation, and AKT-mediated *Foxo-1* (*FOXO1A*) phosphorylation and nuclear exclusion. Beta-cell function and survival

may be regulated through an interplay between *TCF7L2* and *GLP1R/GIPR* expression and signaling in T2DM **(Shu et al., 2009**).

In Sudan, a case control study was performed on 240 T2D cases and 128 unrelated healthy controls to look for associations between T2D and single nucleotide polymorphisms (SNPs) in a number of the top candidate genes in a selected Sudanese population. SNP genotyping was performed using the Sequenom MassARRAY® system. Fourteen SNPs were selected across 7 genes: *CAPN10* (rs2975760 and rs5030952), *PPARG* (rs17036314 and rs1801282), *IGF2BP2* (rs4402960 and rs1470579), *CDKAL1* (rs9465871), *HHEX* (rs1111875), *TCF7L2* (rs7903146, rs11196205 and rs12255372), and *KCNJ11* (rs5215, rs1800467 and rs5219).

The authors found significant associations between the SNPs rs7903146 (odds ratio 1.69) and rs12255372 (odds ratio 1.70) at *TCF7L2* and T2D. The strongest haplotype association (odds ratio 2.24) comprised the two point haplotype T-C across rs7903146 and rs11196205. Adjusted analyses also provided support for protection from T2D associated with minor alleles at SNPs rs2975760 at *CAPN10* (odds ratio 0.44) and rs1111876 at *HHEX* (odds ratio 0.60) **(Ibrahim et al., 2016**).

In the United Arab Emirates, a case control study was carried out to confirm the association of variants rs10885409 of *TCF7L2* and *Pro12Ala* (rs1801282) of *PPAR-γ2* with risk of T2DM and related complications in Emirati population of Arab origin. The study also investigated the interaction of these associations with obesity status. The study was performed on 272 T2DM patients and 216 non-diabetic Emiratis. Genotyping for rs10885409 (*TCF7L2*) and rs1801282 (*PPAR-γ2 P12A*) variants was accomplished with a TaqMan assay. The subgroups were divided according to their obesity status. In the non-obese group, the rs10885409 C allele in the recessive model was significantly associated with the incidence of T2DM (OR 1.975 [95% CI 1.127–3.461]), but this association was not observed in the obese group or when BMI was not considered. *PPAR-γ2* risk allele Pro12 frequency (0.96) was similar in the groups tested, and more than 90% of the study population was homozygous for this allele **(Al-Safar et al., 2015**).

In Saudi Arabia, a study was performed to evaluate the association of single nucleotide polymorphisms (SNPs) rs7903146, rs12255372 and rs4506565 in the documented T2DM susceptibility gene, *TCF7L2* with T2DM among the population of the Eastern Province of Saudi Arabia. The study was performed on 359 T2DM patients and 351 age and sex-matched normoglycemic controls. Genotyping was done by allele specific PCR assay. The results revealed a strong association between risk T alleles in variants rs12255372 (OR: G/T=1.4233; T/T=2.0395) and rs4506565 (OR: A/T=1.6066; T/T=3.1301) and T2DM. However, a common variant, rs7903146, often found to be associated with T2DM in other populations failed to demonstrate any association to T2DM. These data further strengthen the hypothesis that Saudi populations might carry distinct risk alleles in the T2DM susceptibility gene *TCF7L2* (**Acharya et al., 2015**).

2.6.3 Solute carrier family 30, member 8 (*SLC30A8 rs13266634*)

2.6.3.1 Location of gene

SLC30A8 gene contains 8 exons and spans 37 kb. The gene is located on the long (q) arm of human chromosome 8 at position 24.11 (8q24.11) (**Chimienti, Devergnas, Favier, & Seve, 2004**).

2.6.3.2 Gene Function

Zinc functions as a cofactor for numerous enzymes, nuclear factors, and hormones and as an intra- and intercellular signaling ion. Members of the zinc transporter *(ZNT)/SLC30* subfamily of the cation diffusion facilitator family, such as *SLC30A8*, permit cellular efflux of zinc (**Chimienti et al., 2004**).

2.6.3.3 Role in T2DM

The link between impaired β-cell function and Zn transport activity by *SLC30A8* has been reported in several studies. The consensus is that *SLC30A8* is crucial for insulin processing and secretion, and the major contribution of the *SLC30A8* SNPs to T2D is mediated through defects in insulin secretion rather than action. The *SLC30A8* gene encodes the ZnT-8 zinc transporter, which is exclusively expressed in pancreatic β-cells and co-localized with insulin-containing secretory granules (**Kanoni et al., 2011**). *SLC30A8* variants impair islet ZnT8 expression,

insulin secretion, or glucose homeostasis (**Palmer et al., 2008**). In addition, these variants are associated with the production of a less active zinc transporter protein, suggesting less efficiency of zinc accumulation and insulin crystallization. ZnT-8 is thought to be a key protein for insulin secretion by regulating the homeostasis of zinc, which is an essential metal ion for insulin storage and secretion into intracellular vesicles (**Nicolson et al., 2009**).

2.6.4 CDK5 Regulatory Subunit-Associated Protein 1-Like 1 (*CDKAL1* rs10946398)

2.6.4.1 Location of gene

The gene is located on the short (p) arm of chromosome 6 at position 22.3 (6p22.3) of human chromosome and comprises 9 exons **(Zhou et al., 2014)**.

2.6.4.2 Gene Function

By expressing the human and mouse enzymes in *E. coli*, *CDKAL1* has been shown to be a methylthiotransferase that converts N(6)-threonyl-carbamoyladenosine into 2-methylthio-N(6)-threonyl-carbamoyladenosine on *E. coli* tRNA-lys. **(Arragain et al., 2010)**.

2.6.4.3 Role in T2DM

CDK5 is a small serine/threonine protein kinase recognized as an essential molecule in the brain and has several extra-neuronal effects **(Rosales & Lee, 2006)**. CDK5 has been shown to blunt insulin secretion in response to glucose and to play a permissive role in the decrease of insulin gene expression that results from glucotoxicity, as well as in the pathophysiology of β-cell dysfunction and predisposition to type 2 diabetes **(Ubeda, Rukstalis, & Habener, 2006)**. Thus, one can speculate that reduced expression of *CDKAL1* would result in enhanced activity of CDK5 in β cells, which would lead to decreased insulin secretion. In agreement with this speculation, this locus was significantly associated with small decreases in insulin response to a glucose load (**Steinthorsdottir et al., 2007; Saxena et al., 2007; Pascoe et al., 2007; Palmer et al., 2008; Stancáková et al., 2008**).

In Tunisia a study was performed to investigate the association of the rs7756992 of *CDKAL1* and the rs4402960 of *IGF2BP2* with T2DM, diabetic

complications (nephropathy, retinopathy and cardiovascular disease), obesity and hypertension. The study included 200 T2DM patients and 208 controls. Genotyping was performed using TaqMan technology. A significant association between the rs4402960 and T2DM (OR = 1.86) has been found. Overweight/obese subjects bearing the T-allele have an increased risk to develop T2DM (OR = 2.06). Furthermore, the rs7756992 was found to be associated with the reduced risk of diabetic nephropathy in patients with diabetes (OR = 0.44) **(Lasram et al., 2015)**.

2.6.5 Potassium voltage-gated channel subfamily J member 11 (*KCNJ11* E23K)

2.6.5.1 Location of gene

KCNJ11 is located on 11p15.1, which is the short (p) arm of human chromosome 11 at position 15.1. It is a single exon gene encoding the Kir6.2 protein**.**

2.6.5.2 Gene Function

The *KCNJ11* gene provides instructions for making parts (subunits) of the ATP-sensitive potassium (K-ATP) channel. Each K-ATP channel consists of eight subunits. Four subunits are produced from the *KCNJ11* gene, and the other four are produced from another gene called *ABCC8*.

K-ATP channels are found in beta cells, which are cells in the pancreas that secrete the hormone insulin. The K-ATP channels are embedded in cell membranes, where they open and close in response to the amount of glucose in the bloodstream. Closure of the K-ATP channels in response to increased glucose triggers the release of insulin out of beta cells and into the bloodstream, which helps control blood sugar levels **(Bennett, James, & Hussain, 2010)**.

2.6.5.3 Role in T2DM

Several mutations have been reported for *KCNJ11*, two of them are related to increasing risk of T2DM: rs5219 (C>T) for E23K (a glutamate to lysine substitution at position 23) mutation and rs5215 for I337V (isoleucine to valine substitution at position 337) mutation. Several studies showed that the KK homozygote genotype has most relation with T2DM in Caucasians **(Gloyn et al., 2003)**. Investigations have indicated E23K of *KCNJ11* gene as being responsible for sensitivity reduction of K-

ATP thus, the channel is open for more time and insulin secretion is inhibited **(He et al., 2008; Samadikuchaksaraei et al., 2010)**.

In Omanis, a study was performed to investigate the association of 10 known common gene variants with susceptibility to T2DM on 992 diabetic patients and 294 normoglycemic Omani Arabs by using TaqMan real time PCR. The authors examined the following gene variants: *KCNJ11* (rs5219), *TCF7L2* (rs7903146), *CDKAL1* (rs10946398), *CDKN2A/B* (rs10811661), *FTO* (rs9939609 and rs8050136), *IGF2BP2* (rs4402960), *SLC30A8* (rs13266634) *CAPN10* (rs3792267) and *HHEX* (rs1111875). Results confirmed the association of *KCNJ11* (rs5219), *TCF7L2* (rs7903146), *CDKAL1* (rs10946398) and *CDKN2A/B* (rs10811661) gene variants with susceptibility to T2DM among Omani Arabs. The highest genotype variation % between diabetics and controls was found at *KCNJ11* (2.07%) and *TCF7L2* (1.62%). The study, however, was not able to detect an association of T2DM risk with gene variants of IGF2BP2 (rs4402960), *SLC30A8* (rs13266634), *CAPN10* (rs3792267), *HHEX* (rs1111875). *FTO* (rs9939609 and rs8050136) **(Al-Sinani et al., 2015)**.

2.7 Pharmacogenomics of Antidiabetic Drugs

Nucleotide variations in genes encoding K-ATP channel proteins, such as potassium channel inwardly rectifying subfamily J member 11 (*KCNJ11*) and ATP-binding cassette, subfamily C, member 8 (*ABCC8*), are associated with the onset of neonatal diabetes mellitus. Studies on sulphonylureas (SUs) revealed that these drugs might effectively act in response to the defect induced by *KCNJ11* and *ABCC8* mutations in T2DM patients **(Pearson et al., 2006; Rafiq et al., 2008)**.

Many studies have demonstrated the impact of K23E amino acid substitution on SUs therapeutic effects in a cohort Caucasian patients. The studies have revealed that "K-allele" homozygous carriers had a higher reduction in HbA1c levels after 6 months of therapy than "EE" carriers. Sulfonylureas and repaglinide bind to the sulfonylurea receptor (encoded by *ABCC8*), which then inhibits the function of the potassium channel encoded by *KCNJ11* and causes β-cell depolarization and eventual insulin secretion**.** Several studies have reported that sulphonylureas (and also glinides) are able to ameliorate, in T2DM patients, the defective insulin secretion **(He et al., 2008; Javorsky et al., 2012)**.

Nucleotide variations in *TCF7L2* gene have been widely associated with T2DM onset as well as the effectiveness in SU treatments. TCF7L2 is necessary for maintaining the glucose-stimulated insulin secretion (GSIS) and β-cell survival. Thus, variations in the level of active TCF7L2 in β-cells may play a crucial role in determining a progressive deficit in the insulin secretion as well as in accelerating T2DM progression **(Shu, Sauter, Schulthess, Matveyenko, Oberholzer & Maedler, 2007).** Genetics of Diabetes Audit and Research Tayside Studies (GoDARTS) has also revealed the relationship between these two allelic variants and therapeutic outcomes in T2DM patients treated with sulphonylureas. The GoDARTS study enrolled 901 Scottish T2DM patients carrying rs12255372, homozygotes for TT genotype. Patients were treated with sulphonylureas for 3–12 months and compared to individuals with the GG genotype. The results revealed that the TT patients undergoing early SUs treatment had approximately two-fold higher probability to fail (57% versus 17% for TT versus GG resp). These results were confirmed by another independent study on 101 Slovakian patients. In this study, T2DM patients were supplied SUs for six months **(Pearson et al., 2007; Javorský et al., 2013)**.

In the last decade, *PPARG* polymorphisms—both in coding and regulatory regions—have been largely analyzed for their possible association to pathologic phenotypes, such as T2DM. One of the most studied polymorphisms is Pro12Ala (rs1801282). Studies on diabetic patients have recently demonstrated the association between Ala allele and a stronger reduction of HbA1c and fasting glucose plasma levels after treatment with thiazolidinediones (TZD; such as pioglitazone, troglitazone, and rosiglitazone) **(Kang et al., 2005)**.

Polymorphisms of the zinc transporter solute carrier family 30 member 8 gene (*SLC30A8*), including rs13266634 (973C>T, Arg325Trp) and rs16889462 (974G>A, Arg325Gln) SNPs, were recently reported to be related to T2DM development and importantly, to the repaglinide efficacy in T2DM patients. Patients with rs13266634 CT+TT genotypes showed decreased fasting and postprandial insulin levels compared to patients with CC genotype, while a significant differences in the decreased fasting and postprandial glucose and HbA1c levels were found between T2DM patients with GA and GG genotype of *SLC30A8* rs16888462 SNP.

Since *SLC30A8* is mainly expressed in the pancreatic β-cells and appears to play a critical role during insulin maturation and release, the authors have speculated that *SLC30A8* variations influence the zinc disposition and that K-ATP function, affecting the therapeutic efficacy of repaglinide **(Huang et al., 2010)**.

Chapter 3

Materials and Methods

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3.1 Study design

This is a case control study, in which men suffering from T2D were compared to apparently healthy controls T2D.

3.2 Study Sample

The target population of this study consisted of 200 Palestinian men residing in Gaza strip, 100 with T2D and 100 apparently healthy controls. All participants were between 35 and 50 years old.

3.3 Study location

The study was done in the Genetics Lab. of the Islamic University of Gaza and in the Palestinian Medical Relief Society.

3.4 Exclusion criteria

- Cases and controls who are under 35 and over 50 years old.
- Type 1 diabetic patients or any other types of diabetes.
- Patients with renal disease, liver disease, thyroid disorders or other endocrine or chronic diseases.
- Cases and controls who are on hormone replacement therapy or corticosteroid therapy.

3.5 Ethical considerations

Informed consent was taken from all the subjects who agreed to participate in the study. The objective of the study was fully explained to all participants. The study was approved by the Helsinki ethics committee in Gaza Strip.

3.6 Data collection

3.6.1 Questionnaire interview

A personal interview was used for filling in a questionnaire which is designed for matching the study needs for both cases and controls. The questionnaire included

questions on the personal profile of the study population (e.g., age and education), socioeconomic data: family income, family history of diabetes, BMI, and type of treatment. A copy of the questionnaire is provided in Appendix 1.

3.6.2 Body mass index

Body mass index (BMI) was calculated as the ratio of body weight in Kg/height in square meter. Patients were asked to remove heavy clothes and shoes before measurement of weight and height. Medical balance (Seca Model 762, Germany) was used for weight measurement. People with BMI=18.5-24.9 were considered to have normal weight, people with BMI=25.0-29.9 were classified overweight and people with BMI≥30.0 were considered obese **(WHO, 2012)**.

3.6.3 Blood samples collection and processing

About 6 ml venous blood were drawn from all study participants by venipuncture, under quality control and safety procedures. Two milliliters of the collected blood were placed into sterile ethylene diamine tetra acetic acid (EDTA) tubes for DNA extraction and consequent SNPs genotyping. Two milliliters were delivered in plain tubes and left for a while without anticoagulant to allow blood to clot. The tubes were then centrifuged at 3000 rpm for 10 minutes and the serum was collected into fresh tubes. The obtained sera were used for the determination of, C-peptide, cholesterol, triglycerides, HDL-C, LDL-C. About 2 ml blood were placed into separate EDTA vacutainer tubes to be used for the determination of hemoglobin A1c (HbA1c).

3.7 Materials

3.7.1. Equipment

The present work was carried out in the Genetics lab at the Islamic University of Gaza and in the Palestinian Medical Relief Society.

The major equipments used in the study are listed in Table 3.1.

Table (3.1): The major equipment's used in the study

#	Item	Manufacture
1	Thermal Cycler	BioRad, USA/Biometra
2	Horizontal electrophoresis chambers/tanks	BioRad, Germany
3	Electrophoresis power supply	BioRad, Germany
4	Digital balance	AE adam, USA
5	Vortex mixer	BioRad, Germany
6	UV transilluminator Gel documentation system	Vision, Scie-Plas Ltd, UK
7	Safety cabinet	Heraeus, Germany
8	Microcentrifuge	BioRad, Germany
9	Freezer, refrigerator	ORSO, pharml-spain
10	Micropipettes 0.1-2.5 µl/ 0.5-10 µl/ 5-50 µl/ 20-200 µl/100-1000 µl	Dragon-lab, USA
11	Microwave Oven	L.G, Korea
12	Immulite1000	Siemens, Germany
13	Bio-Rad D-10	USA
14	Respons®91	DiaSys, Germany

3.7.2. Chemicals, Kits and Disposables

Chemicals, kits and disposables that were used in this study are listed in Table 3-2.

Table (3.2): Chemicals, kits and disposables

#	Item	Manufacturer
1	Wizard ® Genomic DNA Purification Kit	Promega (Madison, USA)
2	PCR Go Taq® Green Master Mix	Promega (Madison, USA)
3	Agarose	Promega (Madison, USA)
4	PCR primers	Promega (Madison, USA)
5	Nuclease-free water	Promega (Madison, USA)
6	Ethidium Bromide (EtBr) 10mg/ml	Promega (Madison, USA)
7	DNA molecular size marker (Ladder).	BioLab, New England
8	Ethanol 70%	Sigma, USA
9	Absolute Isopropanol	Sigma, USA

10	Filter tips: 0.1-10 µl/ 5-50 µl/ 20-200 µl/ 100-1000 µl	Labcon, USA
11	Microfuge tubes for PCR - thin wall 0.2 mLcapacity	Sigma, USA
12	Microfuge tubes - 1.5 mL capacity	Sigma, USA
13	EDTA tubes	Hy. Labs. Israel
14	Disposable tips	Labcon, USA
15	C-peptide kit	Siemens, Germany
16	Cholesterol kit	DiaSys, Germany
17	Triglycerides kit	DiaSys, Germany
18	HDL-C kit	DiaSys, Germany
19	LDL-C kit	DiaSys, Germany

3.7.3 PCR primers and restriction enzymes

Genotyping of the five gene SNPs was carried out by PCR-RFLP. The primer sequences were obtained from published studies and are provided in Table 3.3.

The restriction enzymes required for the PCR-RFLP identification of each allele were selected from new England Biolabs database. The enzymatic digestion condition and the length of digested fragments (bp) are shown in Table 3.3.

Table (3.3): PCR primers and their characteristics with restriction enzymes, digestion conditions and the length of digestion products.

SNP	Primer	Primer sequence	RE	The Enzymatic Disgestion conditon	Length of digested fragments (bp)
CDKAL **rs10946398**	Forward	5`-CTGCTTGCTGTTGGGGAAGA -3`	AciI	A 8μL aliquot was digested with 2μL of ACIL restriction enzymes (NEB, UK) at 37^{0}C overnight.	**G allele:** 157 bp
	Reverse	5`-CTCAATGCTGTTCATCAGGCAC -3`			**C allele:** 121+36 bp
TCF7L2 **rs7903146**	Forward	5`-ACAATTAGAGAGCTAAGCAC-3`	RsaI	A 8μL aliquot was digested with 2μL of RSAI restriction enzymes (NEB, UK) at 37^{0}C overnight.	**T allele:** 188 bp
	Reverse	5`-GTGAAGTGCCCAAGCTTCTC-3'			**C allele:** 159+29 bp
SLC30A8 **r13266634**	Forward	5`-GAAGTTGGAGTCAGAGCAGTC-3`	HpaII	A 10μL aliquot was digested with 0. 2μL of HpaII restriction enzymes (NEB, UK) at 37^{0}C overnight .	**T allele:** 256 bp
	Reverse	5`-TGGCCTGTCAAATTTGGGAA-3`			**C allele:** 176 + 80 bp
PPRAG **Pro12Ala**	Forward	5`-CAAGCCCAGTCCTTTCTGTG-3`	HpaII	A 10μL aliquot was digested with 0. 2μL of HpaII restriction enzymes (NEB, UK) at 37^{0}C overnight .	**G allele:** 247 bp
	Reverse	5`-AGCTATGACCAGTGAAGGAATCGCTTTCC-3`			**C allele:** 217 + 30 bp
KCNJ11 **E24K**	Forward	5`-GACTCTGCAGTGAGGCCCTA-3`	BanII	A 10μL aliquot was digested with 0. 2μL of BanII restriction enzymes (NEB, UK) at 37^{0}C overnight .	**E:** 150,32,28 bp
	Reverse	5`-ACGTTGCAGTTGCCTTTCTT -3`			**K:** 178,32bp **EK:**178,150,32,28 bp

3.8 Biochemical analysis

3.8.1 Determination of serum C-peptide

The level of C-peptide was measured using enzyme-linked immunosorbent assay (ELISA) **(Andersen, Dinesen, Jorgensen, Poulsen, & Roder, 1993).**

3.8.2 Determination of serum cholesterol

Enzymatic colorimetric method was used for the quantitative determination of total cholesterol in serum **(Meiatlini, prencipe, Bardelli, Giannini, & Tarli, 1978)**.

3.8.3 Determination of serum triglycerides

Enzymatic colorimetric method was used for the quantitative determination of triglycerides in serum **(Bucolo & David, 1973)**.

3.8.4 Determination of serum high density lipoprotein cholesterol

Liquid high density lipoprotein cholesterol (HDL-C) precipitant was used for the determination of HDL-C **(Grove, 1979)**.

3.8.5 Determination of serum low density lipoproteins cholesterol

Serum low density lipoproteins cholesterol (LDL-C) was calculated by using the empirical relationship of Friedewald **(Grove, 1979)**.

LDL-C = Total Cholesterol - HDL-C - TG/5

3.8.6 Determination of HbA1c

Glycated hemoglobin was determined by the colorimetric determination of glycated hemoglobin in whole blood using Stanbio Kit, Texas-USA **(Trivelli et al., 1971)**.

3.9 Genotyping

3.9.1 DNA extraction and polymorphism determination

Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer instructions. The isolated DNA was stored at -20C◦ until analysis.

3.9.2 PCR primers reconstitution

Primers were received in a lyophilized state. Primer containers were first centrifuged at 14,500 rpm for 3 minutes, and then reconstituted with ultrapure nuclease-free water to create a stock solution of each primer with a final concentration of 100 pmol/µl. The stock primer solution was then vortex mixed. Thirty microliter aliquot was taken from stock primer and diluted with 270 µl nuclease free water to make 10 pmol/µl working solution.

3.9.3 Determination of genes polymorphisms

Polymorphisms of the five genes were genotyped using PCR-RFLP. In this technique, two primers -reverse and forward - were used to amplify the gene fragments encompassing the desired SNP. The specific PCR primers sequences were obtained from published studies **(Pascoe et al., 2007; Ciccacci et al., 2012; Arvind et al., 2013; Abdelhamid et al., 2013; Flannick et al., 2014)**. Primers sequences and the expected size of PCR products are listed in Table 3.3. The reactions were carried out in one tube per each SNP. The final volume for each PCR reaction was 20µl, and the reaction components were as described in Table 3.4. Microfuge tubes were then placed in a thermal cycler and PCR amplification was started according to the programs provided in Table 3.5. The sizes of PCR products were visualized under ultraviolet light with a 50 or 100 bp ladder DNA after agarose gel electrophoresis and staining with ethidium bromide.

PCR products were then digested with the appropriate restriction enzymes. Reaction conditions were set as recommended by the manufacturer. Restriction

fragments were resolved on 2% agarose gels along with a 50 or 100 bp ladder DNA and the results were interpreted as depicted in Table 3.3.

Table (3.4): PCR components for amplification of the five genes polymorphisms

Reagent	Volume (µl)	Final concentration
Forward Primer	2	10 pmol
Reverse Primer	2	10 pmol
Nuclease free water	4	-
PCR Go Taq® Green Master Mix	10	1X
DNA sample	2	100ng

Table (3.5): PCR amplification programs

SNP	No of Cycles	Temperature	Time
CDKAL rs10946398 ***SLC30A8 r13266634***	1	94	3 min
	35	94	30 sec
		58	30 sec
		72	40 sec
	1	72	5 min
		4	∞
TCF7L2 rs7903146	1	94	3 min
	35	94	30 sec
		59	30 sec
		72	40sec
	1	72	5min
		4	∞
PPRAG Pro12Ala	1	94	3min

	35	94	30sec
		63	30sec
		72	40sec
	1	72	5min
		4	∞
KCNJ11 E23K	1	94	3min
	35	94	30sec
		62	30sec
		72	40sec
	1	72	5min
		4	∞

3.9.4 Agarose gel electrophoresis (3.0%)

1. Dried agarose gel (2.4 gm) was dissolved in 80 ml 1x Tris-Acetate-EDTA buffer (2M Tris base 1M Glacial Acetic Acid, 0.05 M EDTA) by heating in microwave.
2. Then 3.0 µl Ethidium Bromide (10 mg/ml) was added and mixed, the gel was casted into a mold, which was fitted with a well-forming comb.
3. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus.
4. After amplification, the PCR products and a DNA ladder size marker (Promega, Madison, WI, USA) were loaded into the sample wells to aid in fragment size determination.
5. PCR fragments were detected by size in the agarose gel. Electrophoresis was performed by using Electrophoresis power supply (BioRad, USA) at 70 volts for 40 min at room temperature, and the DNA bands were visualized and documented using a UV trans-illuminator documentation system.

3.9.5 Statistical analysis

Data were analyzed with the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± SD or percentages. The clinical and laboratory characteristics of the T2D patients and the controls were compared with the unpaired Student's t test or One-Way ANOVA test as appropriate. The test for Hardy–Weinberg equilibrium and comparison of genotype and allele frequencies in the T2D patients and the controls were performed using using the Chi square test. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated, using the Calculator for Confidence Intervals of Odds ratio. P-values of less than 0.05 were considered significant.

Chapter 4
Results

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Results

4.1. PCR-RFLP Genotyping Results

The following figures **(4.1** through **4.5)** illustrate in a respective manner, genotyping examples for the "*PPARG, TCF7L2, SLC30A8, CDKAL1*, and *KCNJ11*" genes' polymorphisms investigated in this study.

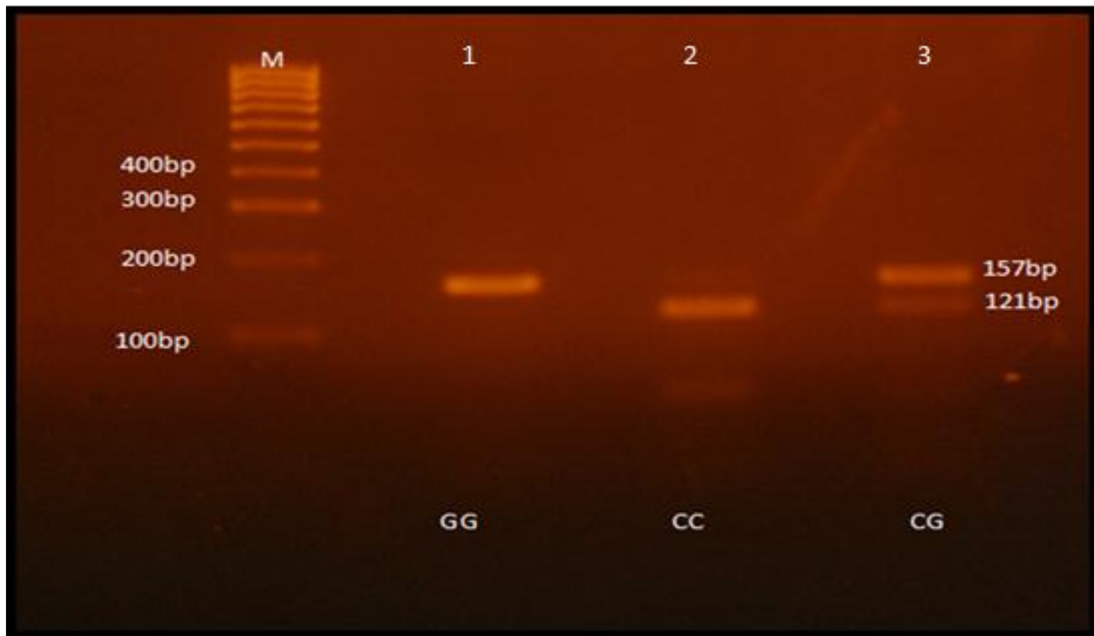

Figure (4.1): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *CDKAL1* gene polymorphism. Lane M: 100 bp DNA ladder. Lane 1 indicates homozygous GG (157 bp), lane 2 indicates CC genotype (121+36bp), and lane 3 indicates a heterozygous 'CG" genotype (157 +121+ 36 bp).

Figure (4.2): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *PPARG* gene polymorphism. Lane M: 100 bp DNA ladder, lanes 1,2,7-10 indicate homozygous CC (217+30bp) genotypes, and lanes 4,5 indicate heterozygous CG samples (217+ 247 + 30 bp).

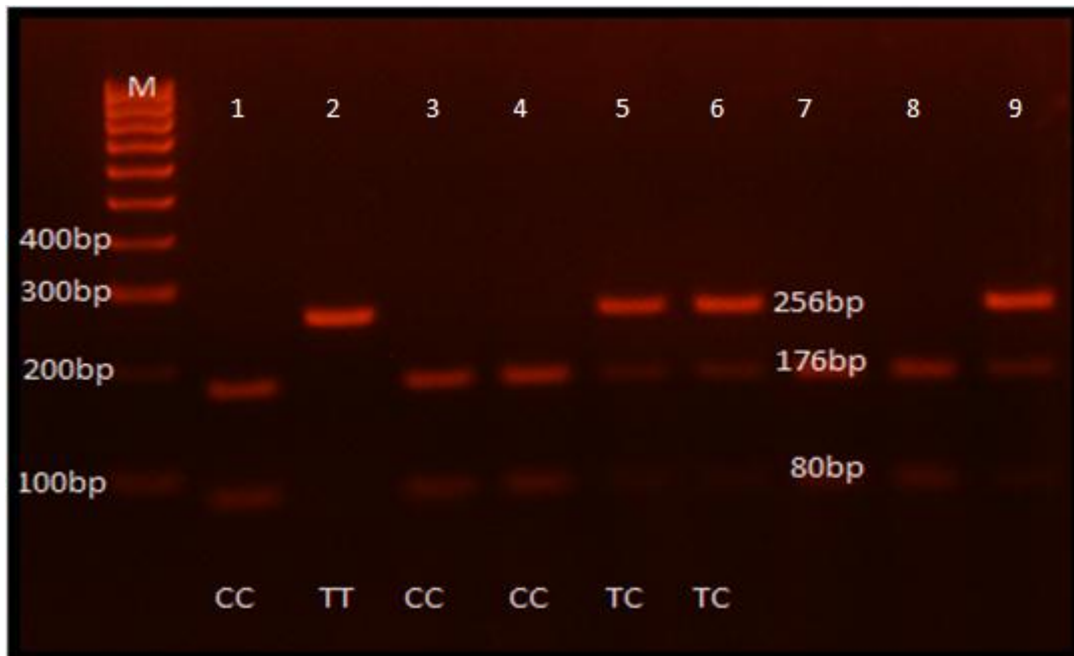

Figure (4.3): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP results of *SLC30A8* gene polymorphism. Lane M: 100 bp DNA ladder, lanes 1,3,4,7,8 indicate homozygous CC (176 + 80 bp) samples, lane 2 indicates a homozygous TT, and lanes 5,6,9 indicate heterozygous TC samples (256+ 176 + 80 bp).

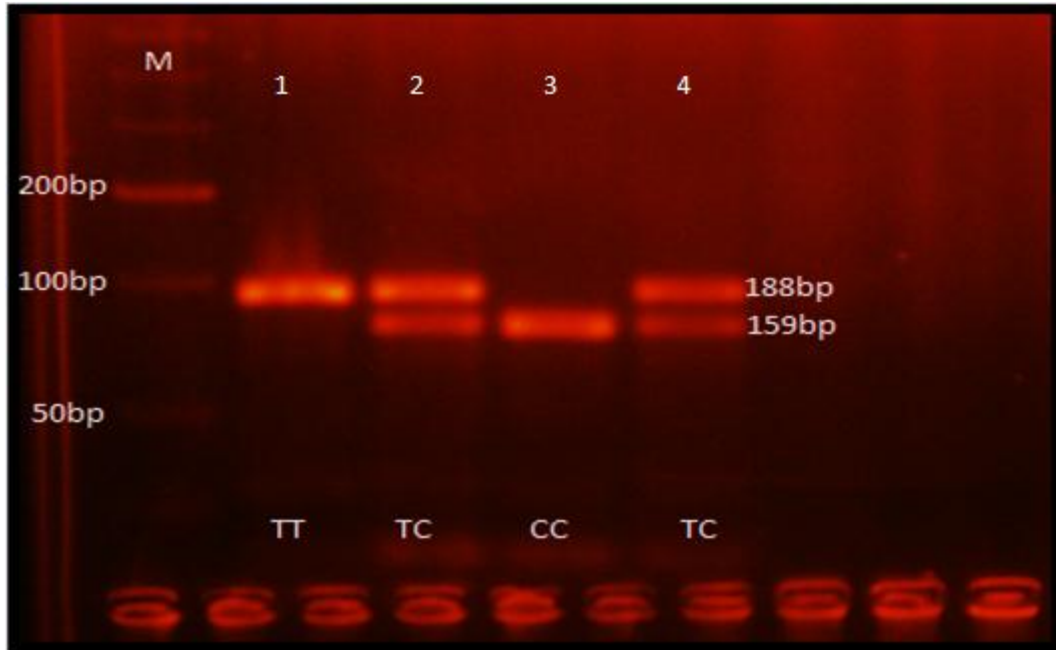

Figure (4.4): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *TCF7L2* gene polymorphism. Lane M: 50bp DNA ladder, lane 1 indicates a homozygous TT (188 bp), lane 3 indicates a homozygous CC genotype (159 + 29 bp), lanes 2,4 indicate heterozygous TC (188 +159 + 29 bp) samples.

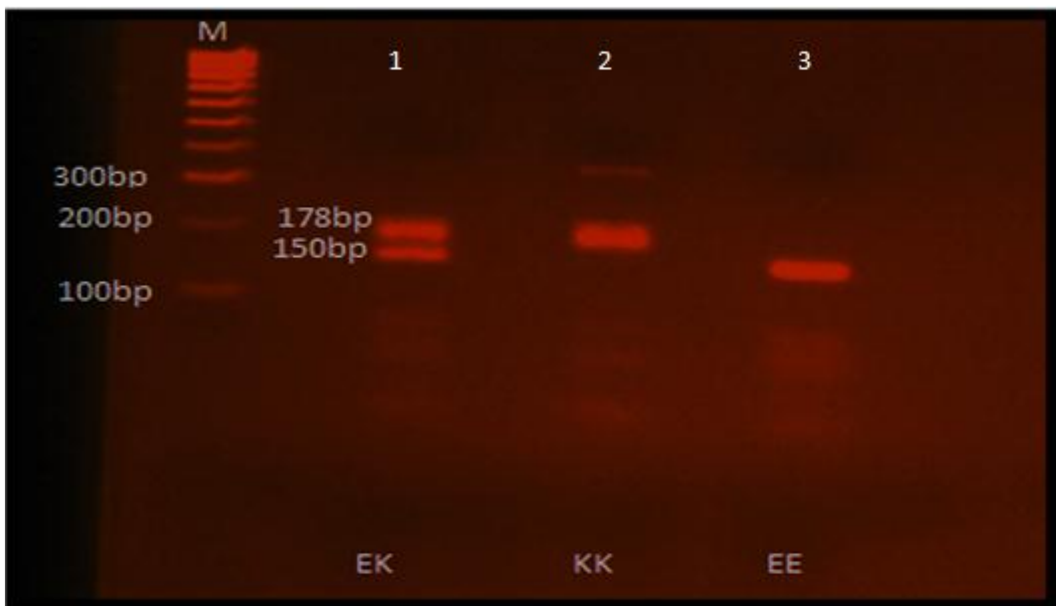

Figure (4.4): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *KCNJ11 E24K* gene polymorphism. Lane M: 100bp DNA ladder, lane 1 indicates a heterozygous EK (178 +150 + 28 bp), lane 2 indicates a homozygous KK genotype (178 bp), and lane 3 indicates a homozygous EE (150 + 28 bp).

4.2 Genotype and allele frequencies of "*PPARG, TCF7L2, SLC30A8, CDKAL1 and KCNJ11*" genes' polymorphisms in patients and controls

Table 4.1 illustrates genotypes and alleles frequencies, odds ratios, 95% confidence intervals and *P* values for the five "*PPARG, TCF7L2, SLC30A8, CDKAL1* and *KCNJ11* genes' polymorphisms among T2D patients and controls. Statistical analyses of genotypic and allelic frequencies for the tested SNPs revealed **no significant** (all P values are > 0.05) difference between T2D patients and controls in four of the tested genes polymorphisms (*PPARG, TCF7L2, SLC30A8, CDKAL1*). *KCNJ11 "E24K"* polymorphism revealed a **significant** (P value is < 0.05) difference between T2D patients and controls. The *KCNJ11* KK genotype and the K allele were significantly (P < 0.05) more frequent in the patient group.

Table (4.1): Genotypes and alleles frequencies of "*CDKAL1, TCF7L2, SLC30A8, PPARG* , and *KCNJ11*" genes polymorphism in the study groups.

SNP	Allele	Patients	Controls	Odds Ratio (95% CI)	P-Value
CDKAL1* *rs10946398	GG	44 (44%)	47 (47%)	0.92 (0.51 to 1.61)	0.77
	CG	44 (44%)	48 (48%)	0.88 (0.50 to 1.54)	0.66
	CC	12 (12%)	5 (5%)	2.59 (0.88 to 7.65)	0.08
	Normal G	132 (66%)	142 (71%)	0.79 (0.52 to 1.21)	0.28
	Mutant C	68 (34%)	58 (29%)		
SLC30A8* *r13266634	CT	38 (38%)	42 (42%)	0.85 (0.48 to 1.49)	0.56
	CC	55 (55%)	53 (53%)	1.08 (0.62to 1.89)	0.78
	TT	7 (7%)	5 (5%)	1.43 (0.44 to 4.67)	0.55
	Normal C	148 (74%)	148 (74%)	1.00 (0.64 to 1.57)	1.00
	Mutant T	52 (26%)	52 (26%)		
TCF7L2* *rs7903146	CT	52 (52%)	47 (47%)	1.22 (0.70 to 2.13)	0.48
	CC	23 (23%)	35 (35%)	0.55 (0.30 to 1.03)	0.06
	TT	25 (25%)	18 (18%)	1.51 (0.77 to 3.00)	0.23
	Normal C	98 (49%)	117 (58.5%)	0.68 (0.46 to 1.01)	0.06
	Mutant T	102 (51%)	83 (41.5%)		
PPRAG* *Pro12Ala	CG	12 (12%)	11 (11%)	1.10 (0.46 to 2.63)	0.82
	CC	88 (88%)	89 (89%)	0.91 (0.38 to 2.16)	0.82
	GG	0.0 (0.0%)	0.0 (0.0%)	1.0 (0.02 to 50.89)	1.00
	Normal C	188 (94%)	198 (94.5%)	0.91 (0.39 to 2.12)	0.83
	Mutant G	12 (6%)	11 (5.5%)		
***KCNJ11* E23K**	EK	35(35%)	35(35%)	1.00 (0.56 to 1.79)	1.00
	EE	53(53%)	62(62%)	0.69 (0.39 to 1.21)	0.20
	KK	12(12%)	3(3%)	4.41 (1.20 to 16.14)	**0.03**
	Normal E	141 (49%)	159 (79.5%)	0.62 (0.39 to 0.97)	**0.04**
	Mutant K	59 (29.5%)	41 (20.5%)		

4.3 The frequencies, odds ratios, and P-values of the three *KCNJ11* E23K genotypes among T2D patients and control subjects under recessive, dominant and co-dominant models.

Table 4.2 illustrates the frequencies, odds ratios, and P-values of the *KCNJ11 E23K* genotypes among T2D and control subjects under recessive, dominant and co-dominant models. The statistical analyses showed that there is a **significant** difference between the two groups under the recessive model. In contrast, the dominant and co-dominant models were **not significantly** different between the two groups.

Table (4.2): The frequencies, odds ratios and P-values of the *KCNJ11 'E23K'* gene polymorphism among T2D patient and control subjects under recessive, dominant and co-dominant models.

SNP	model	Allele	Patients	Controls	Odds Ratio (95% CI)	P-Value
***KCNJ11* E23K**	recessive model	EK+EE	88 (88%)	97 (97%)	0.23 (0.06 to 0.83)	0.03
		KK	12 (12%)	3 (3%)		
	dominant model	EE	53 (53%)	62 (62%)	0.69 (0.39 to 1.21)	0.20
		KK+EK	47 (47%)	38 (38%)		
	co-dominant model	mt/wt EK	35 (35%)	35 (35%)	1.00 (0.56 to 1.79)	1.00
		wt/wt EE + mt/mt KK	65 (65%)	65 (65%)		
mt; mutant type, wt; wild type.						

PPARG, TCF7L2, SLC30A8, CDKAL1 genes polymorphisms were **not significantly** different between the two groups under recessive, dominant, or co-dominant models.

4.4 Hardy-Weinberg equilibrium in the "*PPARG, TCF7L2, SLC30A8, CDKAL1 and KCNJ11*" genes' polymorphisms genotypes

Deviation from Hardy-Weinberg equilibrium (HWE) was assessed as given in the following representative example for *KCNJ11* E23K genotypes:

Frequency of major allele E (p) = (62*2 +35*1)/100*2 = 0.79

Frequency of minor allele K (q) = (3*2 +35*1)/100*2 = 0.21

<u>Expected genotype frequencies:</u>

Genotype EE: $(p)^2$ *100 = $(0.79)^2$ * 100 = 62.41 individuals.

Genotype EK: (2pq) *181 = 2 * 0.79 * 0.21* 100 = 33.1 individuals.

Genotype KK: $(q)^2$ *181 = $(0.21)^2$ * 100 = 4.4 individuals.

The difference between observed and expected genotype frequencies in the control group was determined by using Chi (X^2) square test.

Table 4.3 illustrates the observed and expected genotypes frequencies of "*CDKAL1, TCF7L2, PPARG, SLC30A8, and KCNJ11* " genes' polymorphisms in the control group. Genotypes frequencies did not deviate from Hardy-Weinberg equilibrium expectations. Chi square testing showed that there is **no significant** deviation from Hardy-Weinberg equilibrium for *PPARG, TCF7L2, SLC30A8, CDKAL1 and KCNJ11* SNPs genotypes (all P-values > 0.05).

Table (4.3): Observed and expected genotype frequencies of the "*PPARG, TCF7L2, SLC30A8, CDKAL1 and KCNJ11*" genes polymorphisms in the control group.

	Observed Genotype	**Expected Genotype**	**P-Value**	**Chi Square (X2)**
CDKAL1 rs10946398				
CG	48	41.18	0.10	2.74
CC	5	5.4		
GG	47	50.41		
TCF7L2 rs7903146				
CT	47	48.55	0.75	0.10
CC	35	34.22		
TT	18	17.22		
SLC30A8 r13266634				
CT	42	38.48	0.36	0.84
CC	53	54.76		
TT	5	6.76		
PPRAG Pro12Ala				
CG	11	10.40	0.56	0.34
CC	89	89.30		
GG	00	0.3		
***KCNJ11* E23K**				
EK	35	35	0.46	87.48
EE	62	62		
KK	3	3		

4.5 Comparative analyses of the investigated parameters in patients and controls

As indicated in Table 4.9, there was a **significan**t increase in the means of BMI, triglycerides and HbA1c in patients as compared to controls (P values= 0.00, 0.02, and 0.00, respectively). No significant difference was evident between patients and controls in terms of the rest of the measured parameters.

Table 0.4): Comparative analyses of the investigated parameters in patients and controls.

Group Statistics	Group	n	Mean±SD	t-test for Equality of Means	
				t	Sig. (2-tailed)
BMI	Patient	100	30.27±4.60	3.903	**0.00***
	Control	100	27.89±3.98		
C-peptide	Patient	100	1.80±0.86	-1.29	0.20
	Control	100	1.95±0.75		
Cholesterol	Patient	100	191.44±32.27	-0.339	0.74
	Control	100	193.10±36.75		
Triglycerides	Patient	100	181.39±95.54	2.366	**0.02***
	Control	100	148.50±100.98		
HDL-C	Patient	100	49.55±3.04	-0.286	0.78
	Control	100	49.68±3.39		
LDL-C	Patient	100	109.80±32.24	-1.203	0.23
	Control	100	115.45±34.10		
HbA1c	Patient	100	8.46±1.73	17.447	**0.00***
	Control	100	5.40±0.31		

* P Value is significant at the ≤ 0.05 level.

4.6 The relation between *PPARG, TCF7L2, SLC30A8, CDKAL1* and *KCNJ11* polymorphisms and the tested parameters

The clinical parameters (BMI, C-peptide, lipid profile and HbA1c) were analyzed in the study subjects with respect to the different genotypes of the *PPARG, TCF7L2, SLC30A8, CDKAL1 and KCNJ11* genes' polymorphisms. The analyses results are presented in Tables 4.4 to 4.9.

Table 4.4 showes that the *CDKAL1* (*rs10946398* G>C) genotypes (CC, CG, and GG) have **no significant** effect on the BMI, C-peptide, lipid profile, or HbA1c in the study groups.

Table (4.5): The relation between *CDKAL1* (***rs10946398*** G>C) genotypes and the investigated clinical parameters.

	Patients (n=100)				**Controls (n=100)**			
Parameter	Geno-type	n	Mean+ SD	P value	Geno-type	n	Mean+ SD	P value
BMI	CG	44	29.34± 4.31	0.10	CG	48	27.68±4.28	0.76
	CC	12	29.57± 5.00		CC	5	29.03±4.67	
	GG	44	31.38± 4.63		GG	47	27.99±3.64	
C-peptide	CG	44	1.83± 0.89	0.52	CG	48	1.86±0.73	0.51
	CC	12	2.02± 0.94		CC	5	2.11±1.19	
	GG	44	1.71± 0.82		GG	47	2.02±0.72	
Cholesterol	CG	44	190.41± 29.39	0.95	CG	48	194.38±34.72	0.48
	CC	12	191.25± 35.91		CC	5	173.60±22.30	
	GG	44	192.52± 34.66		GG	47	193.87±39.84	
Triglycerides	CG	44	191.50± 110.02	0.57	CG	48	146.63±65.02	0.77
	CC	12	161.17± 72.88		CC	5	119.80±33.36	
	GG	44	176.80± 85.50		GG	47	153.47±131.95	
HDL-C	CG	44	49.57± 2.79	1.00	CG	48	49.77±3.18	0.69
	CC	12	49.50+4.06		CC	5	50.80±4.44	
	GG	44	49.55± 3.05		GG	47	49.47±3.53	
LDL-C	CG	44	108.96± 31.72	0.97	CG	48	115.55±37.88	0.53
	CC	12	109.58± 35.89		CC	5	98.80±25.00	
	GG	44	110.70± 32.50		GG	47	117.11±30.81	
HbA1c	CG	44	8.42± 1.65	0.96	CG	48	5.38±0.32	0.85
	CC	12	8.59± 2.09		CC	5	5.46±0.43	
	GG	44	8.46± 1.74		GG	47	5.40±0.30	

Analysis results of the relation between *TCF7L2* (rs7903146 T>C) polymorphism genotypes and the measured clinical parameters are illustrated in Table 4.5 below. The *TCF7L2* "TT" genotype exerts **significant effect** on HDL-C level in both patients and controls (*P* values = 0.01 and 0.04, respectively). Individuals harboring this genotype seem to have a significantly higher plasma HDL-C as compared to carriers of the CC genotype.

Moreover, the "TT" genotype proved to be significantly associated with lower plasma LDL-C in the patient group (P=0.001). **No significant** effect of the *TCF7L2* genotypes on the BMI, C-peptide, total cholesterol, triglycerides, or HbA1c was observed.

Table (4.6): The relation between *TCF7L2* (rs7903146 C>T) gene polymorphism genotypes and the investigated parameters.

	Patients (n=100)				**Controls (n=100)**			
Parameter	Geno -type	n	Mean+ SD	P value	Geno-type	n	Mean+ SD	P value
BMI	CT	52	30.51±4.85	0.87	CT	47	27.89±3.80	0.57
	CC	23	30.01±4.12		CC	35	28.32±4.22	
	TT	25	30.01±4.64		TT	18	27.08±4.04	
C-peptide	CT	52	1.79±0.87	0.40	CT	47	1.88±0.74	0.60
	CC	23	1.65±0.80		CC	35	2.05±0.73	
	TT	25	1.98±0.90		TT	18	1.94±0.83	
Cholesterol	CT	52	189.08±32.36	0.15	CT	47	199.11±40.74	0.20
	CC	23	202.65±34.73		CC	35	191.06±34.48	
	TT	25	186.04±28.32		TT	18	181.39±27.23	
Triglycerides	CT	52	174.69±89.30	0.77	CT	47	154.64±133.48	0.82
	CC	23	189.52±107.33		CC	35	145.86±62.20	
	TT	25	187.84±99.71		TT	18	137.61±56.47	
HDL-C	CT	52	49.90±3.04	**0.01**	CT	47	49.13±3.76	**0.04**
	CC	23	47.91±3.15		CC	35	49.51±2.56	
	TT	25	50.32±2.44		TT	18	51.44±3.35	
LDL-C	CT	52	106.44±30.32	**0.01**	CT	47	119.66±37.81	0.27
	CC	23	126.53±36.43		CC	35	115.53±29.72	
	TT	25	101.41±27.41		TT	18	104.28±30.93	
HbA1c	CT	52	8.41±1.93	0.60	CT	47	5.40±0.30	0.96
	CC	23	8.77±1.49		CC	35	5.39±0.36	
	TT	25	8.29±1.50		TT	18	5.39±0.24	

Statistical analyses of the effect of *SLC30A8* (r13266634 C>T) polymorphism genotypes and the measured parameters are illustrated in Table 4.6. The *SLC30A8* "TT" genotype showed a **significant effect** on BMI in the control subjects (*P* value = 0.01) and on lower HbA1c level in the patient group (P value = 0.02). The *SLC30A8* genotypes did not show any significant effect on the other measured parameters.

Table (4.7): The relation between *SLC30A8* (r13266634 C>T) polymorphism genotypes and the investigated parameters.

	Patients (n=100)				**Controls (n=100)**			
Parameter	Geno -type	n	Mean+ SD	P value	Geno-type	n	Mean+ SD	P value
BMI	CT	38	29.66±4.52	0.38	CT	42	27.63±3.64	**0.01**
	CC	55	30.44±4.51		CC	53	27.58±3.93	
	TT	7	32.20±5.77		TT	5	33.40±3.91	

C-peptide	CT	38	1.75±0.86	0.80	CT	42	1.86±0.77	0.39
	CC	55	1.85±0.91		CC	53	1.99±0.71	
	TT	7	1.69±0.50		TT	5	2.30±0.93	
Cholesterol	CT	38	190.45±31.03	0.97	CT	42	189.95±34.71	0.77
	CC	55	191.93±33.64		CC	53	195.53±39.38	
	TT	7	193.00±32.43		TT	5	193.80±26.99	
Triglycerides	CT	38	165.00±89.41	0.27	CT	42	141.12±61.51	0.67
	CC	55	187.47±97.65		CC	53	156.47±127.27	
	TT	7	222.57±107.11		TT	5	126.00±31.11	
H-DLC	CT	38	49.89±2.45	0.37	CT	42	49.76±3.41	0.97
	CC	55	49.49±3.44		CC	53	49.60±3.48	
	TT	7	48.14±2.41		TT	5	49.80±2.59	
L-DLC	CT	38	109.74±27.54	0.97	CT	42	112.83±33.68	0.81
	CC	55	110.20±34.63		CC	53	117.22±35.30	
	TT	7	107.00±40.87		TT	5	118.60±28.73	
HbA1c	CT	38	8.70±1.77	**0.02**	CT	42	5.42±0.31	0.81
	CC	55	8.51±1.68		CC	53	5.38±0.33	
	TT	7	6.74±0.88		TT	5	5.34±0.15	

Results of the relation between *KCNJ11* E23K and *PPRAG* (rs1801282 C>G) genotypes and the measured parameters are presented in Tables 4.7 and 4.8 respectively.. Statistical analyses revealed **no significant** relation between the genotypes and the tested parameters.

Table (4.8): The relation between *KCNJ11* gene *E23K* polymorphism genotypes and the investigated parameters.

	Patients (n=100)				**Controls (n=100)**			
Parameter	Geno-type	n	Mean+ SD	P value	Geno-type	n	Mean+ SD	P value
BMI	EK	35	30.56±4.51	0.67	EK	35	27.67±4.14	0.64
	EE	53	30.32±4.69		EE	62	28.11±3.92	
	KK	12	29.18±4.70		KK	3	26.10±3.94	
C-peptide	EK	35	1.80±0.84	0.96	EK	35	1.87±0.73	0.59
	EE	53	1.82±0.86		EE	62	2.01±0.77	
	KK	12	1.75±0.99		KK	3	1.71±0.43	
Cholesterol	EK	35	190.74±29.13	0.96	EK	35	189.20±31.27	0.73
	EE	53	191.34±33.29		EE	62	195.35±39.84	
	KK	12	193.92±38.78		KK	3	192.00±37.04	
Triglycerides	EK	35	197.11±109.15	0.30	EK	35	139.20±51.97	0.78
	EE	53	178.42±94.30		EE	62	152.71±121.04	
	KK	12	148.67±35.91		KK	3	170.00±96.14	
HDL-C	EK	35	49.66±3.21	0.96	EK	35	50.60±2.98	0.13
	EE	53	49.47±2.70		EE	62	49.15±3.53	
	KK	12	49.58±4.10		KK	3	50.00±3.61	
LDL-C	EK	35	109.31±29.20	0.86	EK	35	110.79±30.60	0.58

	EE	53	109.03±32.35		EE	62	118.24±36.29	
	KK	12	114.65±41.78		KK	3	112.00±28.84	
HbA1c	EK	35	8.56±1.85	0.87	EK	35	5.35±0.29	0.12
	EE	53	8.44±1.61		EE	62	5.40±0.31	
	KK	12	8.27±2.01		KK	3	5.73±0.55	

Table (4.9): The relation between *PPRAG* (rs1801282 C>G) polymorphism genotypes and the investigated parameters.

	Patients (n=100)				**Controls (n=100)**			
Parameter	Geno-type	n	Mean+ SD	P value	Geno-type	n	Mean+ SD	P value
BMI	CG	12	28.87±5.09	0.27	CG	11	28.17±4.54	0.81
	CC	88	30.46±4.53		CC	89	27.86±3.93	
C-peptide	CG	12	1.75±0.65	0.82	CG	11	2.15±0.93	0.34
	CC	88	1.81±0.89		CC	89	1.93±0.72	
Cholesterol	CG	12	185.42±41.34	0.49	CG	11	181.91±18.50	0.29
	CC	88	192.26±31.04		CC	89	194.48±38.24	
Triglycerides	CG	12	172.17±79.62	0.72	CG	11	143.45±80.34	0.86
	CC	88	182.65±97.83		CC	89	149.12±103.61	
HDL-C	CG	12	50.75±3.25	0.15	CG	11	50.82±2.52	0.24
	CC	88	49.39±2.99		CC	89	49.54±3.46	
LDL-C	CG	12	100.40±34.23	0.28	CG	11	102.13±27.57	0.17
	CC	88	111.08±31.95		CC	89	117.09±34.60	
HbA1c	CG	12	8.66±1.95	0.68	CG	11	5.50±0.29	0.24
	CC	88	8.43±1.71		CC	89	5.38±0.31	

Chapter 5

Discussion

Chapter 5
Discussion

Recent advances in human genetic research have facilitated the identification of genetic alterations conferring susceptibility to common diseases, such as T2DM, from across the entire human genome. Through using a large number of subjects and genome-wide association studies, scientists have identified dozens of T2DM risk loci including *KCNJ11, CDKAL1, TCF7L2,SLC30A8,* and *PPARG.*

5.1 Association between *KCNJ11* E23K polymorphism and T2DM:

The *KCNJ11* gene has attracted considerable attention as a promising T2DM susceptibility gene because of its important role in the regulation of glucose-induced insulin secretion. The results of several large population studies and meta-analyses **(Gloyn et al., 2003; Florez et al., 2007; Sakamoto et al., 2007; Rizvi, Raza, Rahman, & Mahdi, 2016; Isakova et al., 2016)** suggest that certain variants, such as E23K, of this locus contribute to T2DM risk.

Our results also support the notion that E23K polymorphism in *KCNJ11* gene is associated with the risk of developing T2DM. We found a significant relation between E23K variant and T2DM. Our results also showed that the under the recessive model, the KK genotype conferred a risk of T2DM as compared to the EE genotype (p = 0.03). However, this locus polymorphism did not show a significant difference between patients and controls in terms of BMI, C-peptide, lipid profile or HbA1c (Table 4.7).

Contradictory to our results, in an Iranian population, it was reported that *KCNJ11* E23K polymorphism is not associated with susceptibility to T2DM (**Keshavarz et al., 2014**). Similarly, in a Moroccan population, it was shown that E23K is not significantly associated with T2DM risk **(Benrahma et al., 2014)**. Therefore, the different genetic/ethnic backgrounds of the different populations seem to play determinative roles in candidate gene association studies.

5.2 Association between *CDKAL1* (rs10946398 G>C) polymorphism and T2DM:

Although the importance of *CDKAL1* (rs10946398 G>C) as a susceptibility polymorphism for T2DM is well established in various populations, we could not replicate such an association in our investigated group. Furthermore, **no significant** difference between case and control subjects could be established between the *CDKAL1* (rs10946398 G>C) genotypes and BMI, C-peptide, lipid profile or HbA1c.

CDKAL1 (rs10946398 G>C) genotypes were significantly associated with high risk of T2DM and β-cell function. Those associations, however, were stronger in Chinese Hans (Asians) than in individuals of European Ancestry **(Burton et al., 2007; Wu et al., 2008; Chauhan et al., 2010; Wood et al., 2016)**. Therefore, the non-significant association between *CDKAL1* (rs10946398 G>C) genotypes and T2DM observed in this study could be due, among other factors, to the different genetic background of the different populations.

5. 3 Association between *TCF7L2* (rs7903146 T>C) polymorphism and T2DM:

Despite the well-known association between *TCF7L2* rs7903146 variant and T2DM **(Scott et al., 2007; Wen et al., 2010; Daniele et al., 2015; Corella et al., 2016)**, our study revealed only a marginal significance (p=0.058) in the Gaza strip population. The lack of strong association observed here is believed to be due to the modest sample size employed.

From another perspective, several reports emphasized that the rs7903146 TT/TC genotypes carriers show significantly lower LDL-C and HDL-C as compared to the minor allele genotype (CC) carriers **(Kimber et al., 2007; Corella et al., 2013; Prokunina-Olsson et al., 2015)**. In our work, the genotype of the common *TCF7L2* rs7903146 allele (TT) showed significant association with higher HDL-C levels in both patients and controls and with lower LDL-C levels in the T2DM patients (Table 4.5). This indicates that harboring the wild-type alleles (TT) protects against elevation of LDL-C and thus may reduce the risk of developing atherosclerosis and other cardiovascular diseases.

5.4 Association between *SLC30A8* (rs13266634 C>T) polymorphism and T2DM:

One of the gene polymorphisms which is strongly implicated in the development of T2DM and impaired glucose tolerance is *SLC30A8* (rs13266634 C>T), particularly in Europeans and East Asians **(Xu et al., 2011; Jing, Sun, Shen, & Zhu, 2011)**. This single-nucleotide polymorphism leads to a missense alteration replacing an arginine with a tryptophan residue at position at position 325 (p.R325W), with anticipated influence on the activity of this Zn transporter.

In our study, statistical analyses of genotypic and allelic frequencies for the *SLC30A8* (rs13266634) revealed **no significant** difference between T2DM patients and controls in the studied Gaza strip population. This result is congruent with the findings of Mtiraoui et. al. (2012) who also showed a lack of association between this variant and T2D in a Lebanese population **(Mtiraoui et al., 2012).** In the contrary, the *SLC30A8* (rs13266634) polymorphism was linked to T2DM in a Tunisian population.

In the current study and as depicted in Table (4.6), the tested *SLC30A8* polymorphism showed significant association with BMI. Control subjects who harbored the CC/CT genotypes showed **significantly** lower BMI levels as compared to carriers of the minor allele TT genotype. On the other hand, the CC/CT genotypes were **significantly** associated with higher HbA1c levels as compared to the T allele homozygous genotype (TT) in the patient group. Studies on European and Chinese populations showed that the glucose-raising C-allele of *SLC30A8* (rs13266634 C>T) is significantly associated with elevated fasting glucose **(Hu et al., 2010)** and this could explain the association between C allele-containing genotypes with higher HbA1c percentage observed in this study.

5.5 Association between *PPARG* (rs1801282 C>G) polymorphism and T2DM:

Peroxisome Proliferator-Activated Receptor Gamma (*PPARG*) is a master transcriptional regulator of adipocyte differentiation and a canonical target of antidiabetic thiazolidinedione medications. The current study evaluated the potential association of *PPARG* (rs1801282 C>G) polymorphisms in patients with T2DM. The

major finding of the present study was that the *PPARG* (rs1801282 C>G) polymorphism is not significantly associated with T2DM in the examined Gaza strip population. Similar results were reported by other investigators (**Pattanayak, Bankura, Balmiki, Das, & Chowdhury, 2014**) who also reported that *PPARG* (rs1801282 C>G) polymorphism does not predispose to T2DM. In the contrary, some studies have shown a significant association between this polymorphism and T2DM (**e.g., Chauhan et al., 2010; Tripathi et al., 2013**).

Moreover, the GG homozygote genotype was not encountered in any of our investigated subjects. The explanation for the absence of this genotype is mainly due to the uncommon occurrence of the G-allele (minor allele frequency "MAF" ~ 0.06) in our population. Given this G-allele frequency and the sample size of the study it is expected to find less than one individual harboring the GG genotype in the study sample (Tables 4.1 and 4.3).

Analysis of the present study results also indicated that the *PPARG* gene polymorphism does not have significant effect on any of the measured clinical parameters (BMI, C-peptide, lipid profile, or HbA1c). These results are in harmony with those of Pattanayak *et al.* (2014) who also could not establish a significant link between this polymorphism and BMI, C-peptide, lipid profile, or HbA1c **(Pattanayak, Bankura, Balmiki, Das, & Chowdhury, 2014).** However, the study of **(Saleh et al. 2016)** related the *PPARG* (rs1801282 C>G) polymorphism with higher fasting plasma glucose and HbA1c levels in the diabetic patients. In their study, the *PPARG* (rs1801282 C>G) polymorphism was also linked to higher LDL-C (**Saleh et al., 2016**).

Discrepancy between results obtained from different populations is a commonplace in candidate gene association studies. This could be due to many reasons including population genetic variation (background/ethnicity) unrelated to the investigated alleles, presence of nucleotide polymorphism somewhere else in the examined genes, epigenetic alterations and linkage disequilibrium to other sequence variants in the vicinity of the investigated loci.

5.6 Association between BMI, C-peptide, lipid profile and HbA1c and T2DM

5.6.1 Serum lipid profile

In patients with T2DM, triglycerides are often elevated, and HDL-C is often decreased or normal. High levels of plasma triglycerides (TGs) are a risk factor for cardiovascular diseases (CVDs). Additionally, TGs are frequently associated with impaired fasting glucose, impaired glucose tolerance, insulin resistance and metabolic syndrome **(Grundy et al., 2005; Ginsberg, Zhang, & Hernandez-Ono, 2005)**.

A TG/HDL-C ratio of ≥3.5 was previously reported to be highly correlated with insulin resistance and atherogenic dyslipidemia in men. This threshold was also associated with metabolic syndrome **(McLaughlin, 2005)**. The TG/HDL-C ratio is also considered a predictor of myocardial infarction and other CVDs **(Salazar et al., 2013; Urbina, Khoury, McCoy, Dolan, Daniels, & Kimball, 2013)**.

Our study showed that the mean level of triglycerides is **significantly** higher in patients as compared to controls (p=0.02). However, differences in the mean levels of cholesterol, HDL-C and LDL-C between patients and controls were **not significant** (P>0.05). The patients TG/HDL-C ratio is **3.69** and this score represents an average risk for cardiovascular diseases, myocardial infarction and insulin resistance.

5.6.2 HbA1c

Glycated hemoglobin (HbA1c) is an important indicator of long-term glycemic control with the ability to reflect the cumulative glycemic history of the preceding (2 to 3) months. HbA1c has also been regarded as a future risk factor for coronary heart disease (CHD), cardiovascular diseases CVDs, chronic kidney disease (CKD), stroke, and all cause mortality **(Selvin et al., 2010, 2011; Di Angelantonio et al., 2014)**.

Furthermore, it has been suggested that improving glycemic control in T2DM patients may be more important than treating dyslipidaemia for the prevention of both microvascular and macrovascular complications, and can substantially reduce

the risk of cardiovascular events. It has been estimated that reducing the HbA1c level by 0.2% could lower the mortality by 10% **(Vaag, 2006)**. The target HbA1c level for people with diabetes is usually less than 7%. The higher the HbA1c, the higher the risk of having complications related to diabetes. In this study, we also noted that the mean level of whole blood HbA1c was **significantly** higher in patients than in controls (8.46±1.73*versus* 5.40±0.31; P=0.000). Moreover, about 84% of our T2DM patients are not under good glycemic control (HbA1c ≥7%). This result is alarming and points to an increased future risk of CVD and other complications related to T2DM in the investigated population.

5.6.3 C-peptide

C-peptide, a cleavage product of insulin, exerts biological effects in patients with type 1 diabetes mellitus, but its role in T2DM mellitus is controversial Furthermore, the role of C-peptide is not well defined in T2DM, which is considered to be a consequence of the reduced β-cell function superimposed on a condition of insulin resistance. In theory, testing C-peptide for very few years should also give some idea of whether or not beta cells are slowly failing.

If patients C-peptide is significantly very low their beta cells are likely to be dead or dying. If they are young or very recently diagnosed with diabetes of any type, a very low C-peptide value is a good way of diagnosing Type 1 (autoimmune) rather than T2DM. But if patients of T2DM have had the disease for decades, and have not kept their blood sugars at normal levels, they may also have a very low C-peptide test values because they may have killed off their insulin-producing beta cells.

Conversely, A high fasting C-peptide level taken at the same time as a high fasting blood glucose test level means that patients are insulin resistant. If patients of T2DM have a high fasting C-peptide level, it is very likely that patients will be able to control their blood sugar by cutting down the amount of carbohydrates they consume.

In the current study, we noted that C-peptide level was **not significantly** different between T2DM patients and controls (1.80±0.86 *versus* 1.95±0.75 µlU/ml; p=0.20). We also found that all the enrolled T2DM patients have normal fasting C-

peptide levels. This finding could be due to the relatively young age (35-50 year) of the study patients in whose major T2DM complications have not developed yet.

5.6.4 BMI

The majority of people with T2DM are overweight or obese. Worldwide, the proportion of T2DM patients with BMI ≥25 kg/m^2 is estimated to be 36.9 % in men and 38.0 % in women. **(Ng et al., 20140)**.

The results of our study clearly showed that there was a **significan**t increase in the mean of BMI among patients as compared to controls (30.27±4.60 *versus* 27.89±3.98, P=0.00). The majority of our enrolled T2DM patients are overweight and obese. Patients with BMI ≥25 kg/m^2 presented (84%), and those with BMI >30 kg/m^2 constituted (51 %). Overweight or obese patients (BMI ≥25 kg/m^2) have a higher rate of cardiac events (such as the acute coronary syndrome and heart failure) and other chronic diseases **(Costanzo et al., 2015)**.

Despite a strong association between body weight and mortality in the general population, several studies indicate that among patients with T2DM and cardiovascular disease, overweight or obese patients (BMI ≥25 kg/m^2) had a lower mortality as compared to patients with normal weight **(Carnethon et al., 2012; Doehner et al., 2012; Goyal, Nimmakayala, & Zonszein, 2014; Costanzo et al., 2015).** There may be an "obesity paradox" between T2DM and cardiovascular risk. The obesity paradox is a medical hypothesis which holds that obesity may be protective and associated with greater survival in certain groups of people, such as those with certain chronic diseases e.g., T2DM **(Gruberg et al., 2002)**.

Chapter 6

Conclusion & Recommendations

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6.1 Conclusion

The present case-control study focused on the contribution of *KCNJ11* (E24K), *PPARG* (Pro12Ala), *TCF7L2* (rs7903146 T/C), *SLC30A8* (r13266634 C/T), and *CDKAL1* (rs10946398 C/T) polymorphisms to T2DM in Gaza Strip. The study also examined the relation between those polymorphisms and BMI and various biochemical parameters in the study sample. The results of the study can be summarized as follows:

1. *KCNJ11* (E24K) polymorphism proved to be **significantly** associated with T2DM in Gaza strip patients. The K/K genotype was **significantly** higher in the T2DM patients as compared to controls.
2. The *TCF7L2* (rs7903146 T/C) polymorphism showed marginal significance in terms of its association with T2DM risk (p=0.058).
3. Genotypic and allelic frequencies of *PPARG* (Pro12Ala), *SLC30A8* (r13266634 C/T), and *CDKAL1* (rs10946398 C/T) did not differ significantly between T2DM patients and controls.
4. The genotypes of the investigated loci were all in Hardy-Weinberg equilibrium.
5. *CDKAL1* (rs10946398 C/T) polymorphism may influence HDL-C and LDL-C levels. The common allele homozygote (TT) genotypes are significantly associated with higher plasma HDL-C levels whereas, the CC genotype is significantly associated with higher plasma LDL-C levels.
6. *SLC30A8* (r13266634 C/T) polymorphism impacts the BMI. The common allele (C) containing genotypes (CC and CT) are **significantly** associated with lower BMI levels in the control subjects. In contrast, the CC and CT genotypes are **significantly** linked to higher HbA1c percentage.
7. The mean BMI is **significantly** higher in patients as compared to controls. The majority of the investigated T2DM patients are overweight and obese.
8. The mean levels of HbA1c and triglycerides are **significantly** higher in T2DM patients as compared to controls.

6.2 Recommendations

1. Conducting studies on a larger sample to confirm, or rule out, the association between *TCF7L2* (rs7903146 T/C) polymorphism and T2DM in the Palestinian population.
2. Employing *KCNJ11* (E24K) polymorphism testing for predicting the risk of developing T2DM in asymptomatic individuals and for selecting the appropriate therapeutics in T2DM patients.
3. Performing further studies to investigate the role of other documented loci polymorphisms in T2DM in our population.
4. Carrying out a similar study on T2DM female patients in order to reveal combined gender/polymorphism effect, if any.
5. Urging patients to control their HbA1c, LDL-C, triglyceride levels and body weight in order to avoid the complications associated with T2DM.

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Appendix 1

Case control study, questionnaire for Candidate Gene Polymorphisms and Risk of Type-2 Dibetes Mellitus in Gaza Strip.

أخي المواطن/ أرجو مساعدتنا في إتمام هذه الدراسة (بحث ماجستير تحاليل طبية / الجامعة الإسلامية) والتي تختص بترشيح جينات تزيد من خطر الاصابة بمرض السكري من النوع .

Patients and controls Questionnaire

1. Personal profile of the study population:

Name:__________________

Age:___________________

Gender:___________________________

Education **□University or diploma** **□Secondary school**

□Preparatory school **□Primary school**

□Illiterate

2. Socioeconomic data of the study population:

- **Employment:** **□Yes** **□No**
- **Family income: <1000 Shekels …….1000-2000 Shekels ……**
  >2000 Shekels …….

- **Family history for Type 2 diabetes** □**Yes** □**No**
- **Physical activity:** □**Yes** □**No**
- **chronic diseases.(renal disease, liver disease, thyroid disorders or other):**
    - **Meal frequency per day** □**One** □ **Two** □**Three** □ **four and more**

5.Type of treatment .

Agreement:

I agree to complete this questionnaire concerning my health statement.

أنا موافق/ ة على تعبئة هذا الاستبيان الذي يتعلق بصحتي والمشاركة فيه.

التوقيع: ....

التاريخ: .... شكرا لكم على حسن تعاونكم

الباحثة/ اسراء عمر الطلالقه