Deanship of Graduated Studies

Al-Quds University

APOE Gene Variants and Risk of Hyperlipidemia in Palestinian Type 2 Diabetic Mellitus Patients

Manal Mohammed Saleh Ghattas

M.Sc. Thesis

Jerusalem – Palestine

1443/2021

ApoE Gene Variants and

Risk of Hyperlipidemia in Palestinian T2DM patients

Prepared by:

Manal Mohammed Saleh Ghattas

B.Sc. in Biology-major and Medical Laboratory Sciences-minor in

Bethlehem University/ Palestine

Supervisor: Dr. Suheir Ereqat

Cosupervisor: Dr. Abdelmajeed Nasereddin

A Thesis Submitted in Partial Fulfillment of Requirements for the Degree of Master of Biochemistry and Molecular Biology/ Faculty of medicine -Al-Quds-University

1443/2021

Deanship of Graduated Studies

Al-Quds University

Thesis Approval

ApoE Gene Variants and

Risk of Hyperlipidemia in Palestinian T2DM patients

Prepared by: Manal Mohammed Saleh Ghattas

Registration No.: 21810881

Supervisor: Dr. Suheir Ereqat

Cosupervisor: Dr. Abdelmajeed Nassereddin

Master thesis submitted and accepted. Date: 23.1.2021

The names and signatures of the examining committee members are as follows:

1- Head of committee: Dr. Suheir Ereqat Signature: Suheir Ereqat

2- Internal Examiner: Dr. Rula Abdul-Ghani Signature:

3- External Examiner: Dr. Amjad Hussein Signature:

Jerusalem-Palestine

1443/2021

Dedication

To my mother and father, to my husband and daughter, to my family

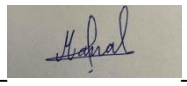
To my teachers

To all supporters

Manal Mohammed Saleh Ghattas

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except there otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: ___  __

Manal Mohammed Saleh Ghattas

Date: 23.1.2021

Acknowledgments

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

I would like to deeply thank and appreciate Dr. Suheir Ereqat, my supervisor for her skillful help and support.

Special thanks to Dr. Abedelmajeed Nassereddin, my co-supervisor for his support and help.

I would like to thank all my teachers in Biochemistry and Molecular Biology department that I have really been well-taught by their hands, especially Dr. Fawwaz Awwad and Dr. Rula Abdul-Ghani.

I would like to thank Mr. Anas Sabarneh for collecting samples from Ramallah hospital. Thanks to Mrs. Lina Salah at the Medical Research Center and to Ahmad Abdel Qader at Al-Abraj laboratory for their help and guidance.

I also would like to thank Dr. Khaled and Dr. Muawiyeh for their help in the laboratory work. Special thanks to Deanship of scientific research for financial support.

I am such a lucky person to have the chance to get to know the really helpful staff in Alquds university and medicine faculty particularly.

Abstract

Diabetes is considered as one of the most popular metabolic diseases worldwide. Type 2 is the most common by which it affects 90% of all diabetic patients in the world. The problem with the disease is that insulin is not doing its job or it is not secreted at all. This elevates the sugar level in the bloodstream, as it is not entering the human cells. There are several contributing agents that participate in the development of T2DM, they could be environmental or genetic or both. Many genes have been shown to be involved in increasing the risk of T2DM. One of these genes is *APOE* gene which is responsible for producing apolipoprotein E that is important in lipid metabolism.

The study is a cross sectional that was done in the period from January to April 2019. It consists of T2DM Palestinian subjects divided into two groups; lipidemic (case) and non-lipidemic (control). A total of 204 diabetic subjects were included in this study; 96 participants were lipidemic and 108 were non-lipidemic. All patients aged over 50 years and admitted in Ramallah hospital/Palestine. The lipid profile was performed during the hospital admission examination. Two SNPs within the exon 4 of the *APOE* gene and three SNPs in the *APOE* promoter region were identified by amplicon based next generation sequencing (NGS) and it is the first to be held on Palestinian population. The frequencies of these polymorphisms were assessed in the studied population. Further, the associations of *APOE* genotype and promoter SNPs with risk of hyperlipidemia in T2DM patients were investigated. Our results showed that the frequency of E3 allele was the highest (79.6%) among other alleles; E3/E3 genotype was also found to be highest in all T2DM subjects with a frequency of 78.1%. Our study population was stratified according to lipid status, the obtained results showed no statistical differences ($P>0.05$) of *APOE* genotypes frequency (*APOE* polymorphisms and promoter SNPs) among lipidemic and non lipidemic groups. Further, Logistic regression analysis adjusted for age, gender and BMI showed no

association between *APOE* genotypes (epsilon and promoter polymorphisms) and risk of dyslipidemia. No significant association was observed between *APOE* genotypes and serum cholesterol, TG, HDL-C and LDL levels. The possible synergistic effect between the promoter SNPs and the common *APOE* polymorphism was also investigated. We found no significant differences between lipidemic and non lipidemic groups after stratification of the promoter SNPs with the *APOE* genotypes.

In conclusion, association between known SNPs and diseases are varied among different ethnic groups. Our study showed no association between *APOE* genotypes and hyperlipidemia in type 2 diabetic patients in the Palestinian population. Further studies should be done on larger sample to confirm the study results.

Contents

List of Tables

List of Figures

Figure No. Title of the figure Page No.

List of appendices

List of abbreviations

Abbreviation	Term
AD	Alzheimer's Disease
APOE	ApolipoproteinE
Arg	Arginine
β	Beta
BMI	Body mass index
Bp	Base pair
Cys	Cysteine
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
FTO	Fat mass and obesity
FBS	Fasting blood sugar
GDM	Gestational diabetes mellitus
GWAS	Genomic wide association studies
HbA1c	Glycohemoglobin A1c
HDL	High density lipoprotein
HGNC	HUGO Gene Nomenclature Committee
ID	Identity document
IRS1	Insulin receptor substrate-1

KCNJ11	Potassium Inwardly Rectifying Channel Subfamily J
LDL	Low density lipoprotein
LPS	Lipopolysaccharides
MAF	Minor allele frequency
µl	Microlitre
M±SD	Mean±standard deviation
NGS	Next generation sequencing
OR	Odd ratio
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PPARG2	Peroxisome proliferator-activated receptor gamma 2
SBP	Systolic blood pressure
SCFAs	Short chain fatty acids
SNP	Single nucleotide polymorphism
SPSS	Statistical product and service soultions
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAE	Tri-acetate EDTA
TC	Total cholesterol
TCF7L2	Transcription factor 7-like 2
TG	Total triglycerides
UV	Ultraviolet light
VDR	Vitamin D receptor
WHO	World health organization
$\chi 2$	Chi square

Chapter one

1. Introduction:

1.1 Diabetes Mellitus:

Diabetes Mellitus (DM) is defined as a group of metabolic diseases resulting from an increase in blood sugar which is called hyperglycemia, and this occurs due to insulin secretion defects or problems that are not in insulin itself but response to it (Kharroubi 2015). Chronic hyperglycemia produces several complications with long-term damages including failure of different organs such as kidneys, liver, eyes, nerves and blood vessels. DM is considered as one of the oldest discovered metabolic diseases known to affect human beings (Baynest, et al. 2015).

1.2 Prevalence of Diabetes Mellitus

The number of people affected with diabetes mellitus is increasing dramatically during the last decades with more than 300 million people worldwide reported by 2018 (Zhen, et al. 2018). In 2020, world health organization (WHO) has announced that 422 million people are affected with diabetes worldwide. The disease prevalence number was predicted to reach 642 million people with DM by 2040 (Ogurtsova, et al. 2017). It is reported that 43% of diabetic patients die each year from the disease and its complications (Ghandour, et al. 2018).

According to WHO, the highest prevalence of DM is known to be in the Mediterranean regions with 13.7% of the population affected (Roglic, et al. 2016).

In Palestine, ministry of health showed 21% of population was affected with diabetes in 2019. And foretold to reach 23.4% by 2030. It is estimated that 5.7% of all deaths in Palestine are due to diabetes and its complications as it is the fifth cause of death (ministry

of health report, 2019: http://www.site.moh.ps/index/ArticleView/ArticleId/4717/Language/). Figure 1.1 shows the prevalence of diabetes worldwide and how much it increased year over year from 1980-2014.

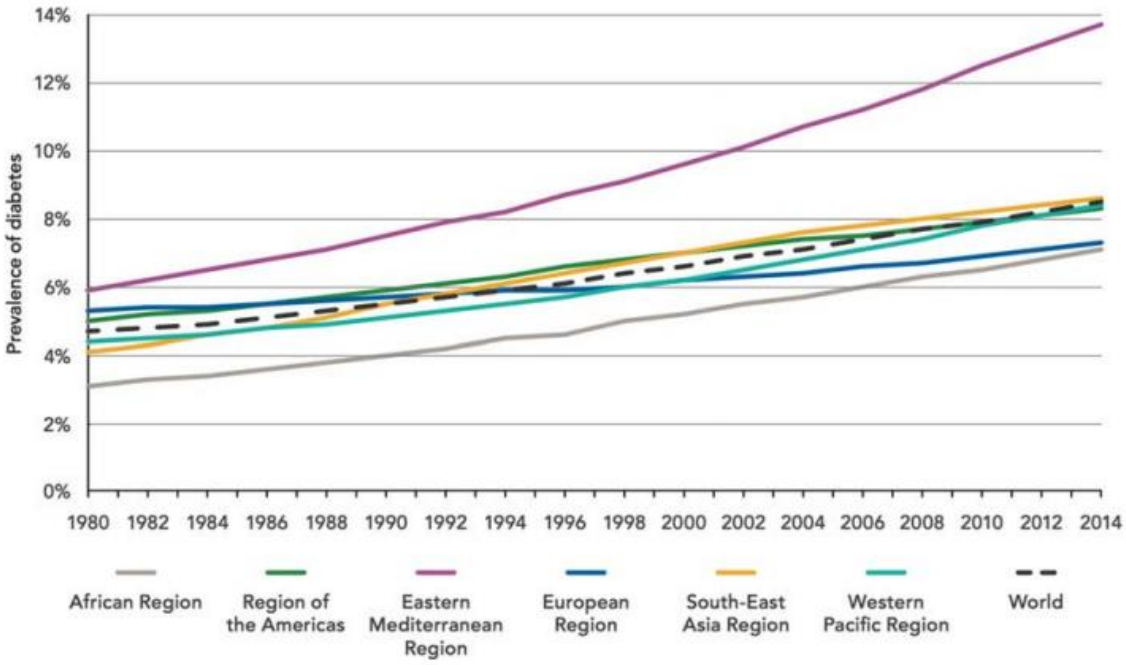


Figure 1.1: Prevalence of diabetes, 1980–2014 (Fan, et al. 2017).

1.3 Classification of Diabetes Mellitus

There are three main types of DM: First, type1 DM that is immune-mediated. Second, type 2 DM characterized by insulin resistance. Furthermore, gestational diabetes that occurs during pregnancy and usually reserves after birth. There are also less common types of diabetes such as cystic fibrosis-related diabetes and monogenic diabetes (Das, et al., 2017)

1.3.1 Type 1 Diabetes Mellitus (T1DM)

It is also called juvenile diabetes and was named previously as insulin dependent diabetes. It results from a deficiency in insulin secretion due to beta cells destruction that produces insulin due to an autoimmune action. The markers of type 1 DM include autoantibodies to insulin, islet cell autoantibodies, and anti–glutamic acid decarboxylase. This type affects

(5-10)% of those who have diabetes and are treated by insulin therapy to maintain normoglycemia (Katsarou, et al. 2017).

1.3.2 Type 2 Diabetes Mellitus (T2DM)

It is referred to non-insulin dependent diabetes or late-onset diabetes. It is the most common type (80%-90%) of all diabetic patients. The problem that it is incorporated with high blood glucose level is insulin resistance which prevents the entrance of glucose to cells. T2DM is considered to be one of the major causes of death worldwide and the predictions estimate that it will be the seventh leading cause of death by 2030. Many genetic and environmental factors contribute to the development of T2DM (Shah, et al. 2017).

1.3.3 Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that is firstly recognized during pregnancy. Approximately 7% of all pregnant women develop gestational diabetes worldwide. The disorder appears during the third trimester of the pregnancy and might continue after birth. The treatment is through diet modification (Kim, et al. 2002) & (Tashrifi, et al. 2018). There are many causes lead to GDM and increasing its occurrence in pregnant women, for example: obesity, childbearing at later age, family history of being affected with T2DM, having GDM in a previous pregnancy, and ethnicity. These are the major reasons for the development of the disease. To diagnose the disease, oral glucose tolerance test (OGTT) is done to the pregnant woman (McIntyre, et al. 2019).

1.4 Clinical Features of Diabetes Mellitus

Symptoms appear to be the same in all types of diabetes with differences in the severity and development rate.

1.4.1 Clinical Features of Type 1 Diabetes Mellitus

Some of the symptoms of T1DM include weight loss, polydipsia, polyurea, polyphagia, constipation, fatigue, blurred vision, cramps, and candidiasis. Long lasting T1DM patients may be susceptible to microvascular and macrovascular complications like heart, coronary artery, and peripheral vascular diseases (Mauricio, et al. 2020).

1.4.2 Clinical Features of Type 2 Diabetes Mellitus

T2DM patients have a high risk of large vessel atherosclerosis which is commonly associated with hyperlipidemia, hypertension and obesity. Most patients die from end stage renal disease and cardiovascular complications (Craig, et al. 2014).

1.5 Diabetes Mellitus Diagnostic Criteria

It was previously considered that HbA1c more than 6.5% is the cut point for diabetes diagnosis (Maureen, et al. 1995). However, WHO considered that HbA1c alone is not a suitable diagnostic test for diabetes. There should be several tests to decide whether a person is affected or not. The following table (1.1) summarizes the 2020 WHO recommendations for the criteria for diabetes diagnosis.

Table1.1: Diagnostic criteria of glucose tolerance categories according to the WHO, 2020.

Measurement	**Diagnostic cut-off value**
Random blood glucose	≥11.1 mmol/L (200mg/dL)
Fasting venous or capillary plasma glucose	≥7.0 mmol/L (126 mg/dl)
Oral glucose tolerance test	≥11.1 mmol/L (200 mg/dL)
HbA1c	≥ 6.5% (48 mmol/mol)

Many tests could be done to indicate the level of sugar in the blood. First, Random blood sugar test; it is a simple test and there is no need for fasting before doing the test. If Glucose level reaches 200mg/dl or higher then it indicates diabetes but should be confirmed with other tests (Amreen, et al. 2018).

Second, fasting plasma glucose test; the person should fast eight hours before doing the test. Glucose level of 126mg/dl or more appears in diabetic patients (Lopez-Lopez, et al. 2018).

Third, oral glucose tolerance test; it shows how the body act when glucose level is high. The person should fast eight hours before doing the test. The fasting blood glucose is measured, then 75 to 100 grams (depending on person's weight) of glucose are ingested and glucose level is tested every 30 minutes within 2 hours. Two-hour blood glucose level of 200mg/dl or more indicates diabetes (Tashrifi, et al. 2018).

Fourth, testing glycated hemoglobin level. The life span of hemoglobin is 90 to 120 days in vivo. Glycated hemoglobin A forms during this time, resulting in the formation of ketoamine compound by combination of hemoglobin A and glucose. Several glycated hemoglobin subfractions have been isolated. Glycated hemoglobin A fraction (HbA1c) is of most interest to indicate the average glucose concentration. The blood HbA1c of 6.5%

or higher is considered as diabetes (Little, et al. 2019). HbA1c is recommended as a significant indicator to monitor blood glucose control (Selvin, et al. 2010). The test can be accomplished anytime the person goes to do it regardless of the content of the meal one has eaten or the duration of fasting (Hu, et al. 2010).

1.6 Correlations with and Influencing Factors on T2DM

There is a combination of genetic and environmental factors contributing to the disease development.

1.6.1 Lifestyle Factor Correlation with Diabetes Mellitus

Different lifestyle factors are of great importance to T2DM development, such as sedentary lifestyle, smoking, alcohol consumption and physical inactivity. Substantial epidemiological studies have reported that the most important risk factor for T2DM is obesity, as it may influence insulin resistance development and the progression of the disease. According to the WHO, nearly 90% of diabetic patients who develop T2DM are mostly obese with excessive body weight (obesity) (Wu, et al. 2014). See figure 1.2 A.

1.6.2 Other Contributing Factors

In addition, many studies showed that gut metagenome is a factor for the development of T2DM as different kinds of gut bacteria could play different roles in maintenance or interaction with their environment (Sircana, et al. 2018). Three years after birth, stabilization of intestinal microbiome occurs. Alteration of the microbiome due to medications and changes in diet leads to changes in the functions they do. In which their composition include lipopolysaccharides (LPS), a bacterial endotoxin that is derived from gram-negative bacterial membrane entering bloodstream, it is been shown to cause inflammation. In addition, short-chains fatty acids (SCFAs) that affect the liver function

ruining the metabolism of gluconeogenesis. The scientists believe this could be the reason for gut microbial role in T2DM development. They have also noticed that diabetic mice and humans have elevated LPS (Aydin, et al. 2018). See figure 1.2 C.

As shown in figure 1.2 D, other contributors that may have a significant role in developing T2DM are vitamin D and K deficiencies that were found in some diabetic patients. Vitamin D receptor (VDR) is present in pancreatic β cells giving vitamin D the characteristic to play a vital role in the synthesis of insulin and its release. Moreover, vitamin D controls the flux of calcium through the membrane of β cells, so it affects insulin sensitivity. While vitamin K has been correlated with higher sensitivity of insulin and greater glycemic status, so vitamin K provides important benefits of glucose homeostasis (Wu, et al. 2014).

All these factors could lead to insulin resistance developing type 2 diabetes mellitus. The mechanism is represented in figure 1.2 E.

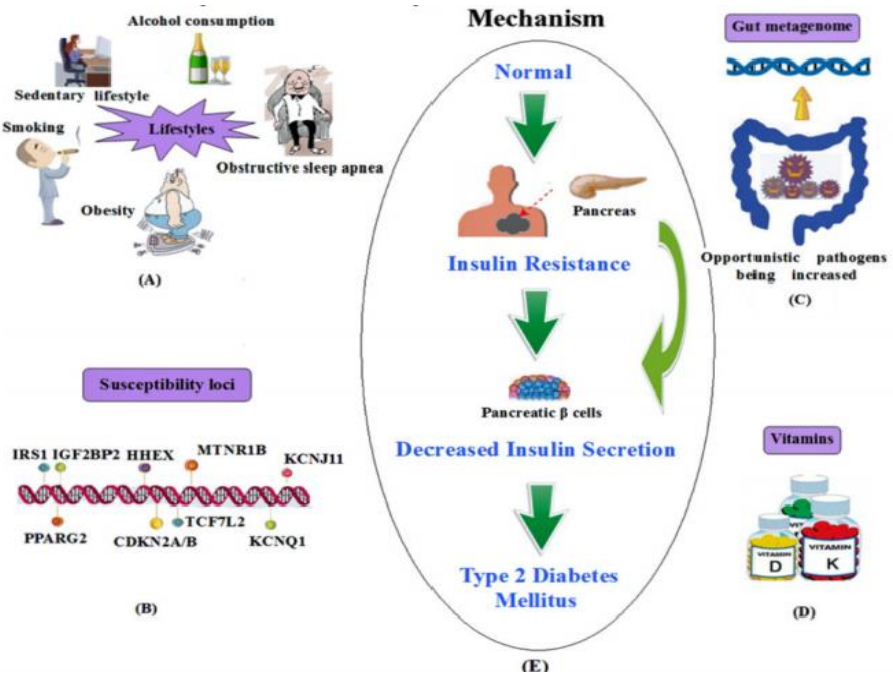


Figure 1.2: Summary of the influencing factors and mechanism of T2DM. (Wu, et al. 2014).

1.7 Correlation of Genetic Factors with Diabetes Mellitus

Segregation analysis in several studies suggests the polygenic nature of T2DM. Genome-wide association studies (GWAS) has discovered the susceptibility loci of T2DM since early 2007. Then, numerous GWAS conducted in many countries and different ethnic groups have reported that there are linkage signals at the same or different chromosomes with T2DM. They identified approximately 75 susceptibility loci which are related to T2DM. Examples of candidate genes are *KCNJ11,TCF7L2 , IRS1, PPARG2, FTO, APOE* and other genes (Ali, 2013).

Genetic issue cannot be controlled; it is there in DNA and will be there long last. A lot of populations genetic studies described the association between several genes and type 2 diabetes mellitus disease. One of these studies is *FTO* gene association with T2DM, Sabarneh and others have found that people with minor allele A of *FTO* gene variant SNP (rs9939609) increase the risk of T2DM (OR=1.84) in Palestinian population (Sabarneh, et al. 2018). The minor allele frequency (MAF) among type 2 diabetic Palestinian population was 58.8% which is higher in comparison with diabetic Chinese people who have 12% MAF (Li, Wu et al. 2008). A meta-analysis study done on Asian and Caucasian population says that Transcription factor 7 like 2 (*TCF7L2*) rs7903146 is correlated with high risk of being affected with T2DM (Lou et al., 2019). Several genes have been studied to be correlated with T2DM like *MTHER, SLMAP, APLN, TNF-A* and *APOE*. Figure 1.3 shows the genes known to be incorporated with Arab T2DM patients. *APOE* is not well-studied in Arab world so it is not represented in the figure.

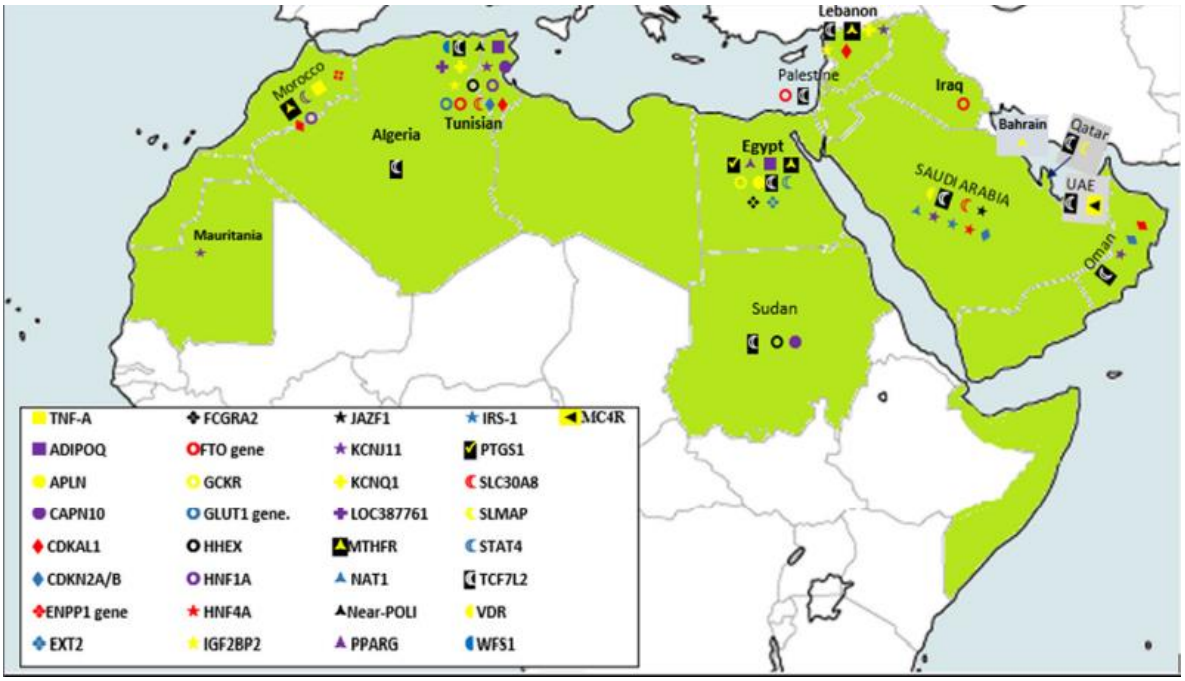


Figure 1.3: Distribution of associated genes with T2DM among Arab countries. (Abuhendi, et al. 2019).

1.7.1 *Apo lipoprotein E* (*APOE*) Gene

It is important to study genetic factors related to diabetes, then you could control the environmental ones reducing the chance to develop the disease.

1.7.2 *APOE* Gene Alleles

The location of *APOE* gene on the chromosome is at position 19q13.2 and there are three common alleles of *APOE* (E2, E3 & E4) produced by two SNPs at positions 112 (rs429358) or 185 (rs7412) giving three isoforms of apoE (ApoE2, ApoE3 and ApoE4) which differ in their binding affinity to apoE receptor. These three isoforms produce six different genotypes (E2,E2/ E2,E3/ E2,E4/E3,E3/ E3,E4/ E4,E4) (van den Bosch, et al. 2019). The most common allele in the world is E3 with high frequency (77.9%), however, the frequency of E2 and E4 is 8.4% and 13.7%, respectively. The single nucleotide differences among the three apoE isoforms produce different amino acids in both locations

112 and 158 where either cysteine or arginine is there: E2 has two cysteine in both sites (Cys 112, Cys 158), E3 has one cysteine and one arginine in each site (Cys 112, Arg 158) and E4 with two arginine in both sites (Arg 112, Arg 158). The structure and function of the three apoE isoforms is different and affect the disease risk (Zhong, et al. 2016). Figure 1.4 shows the schematic diagram of *APOE* gene and its three alleles.

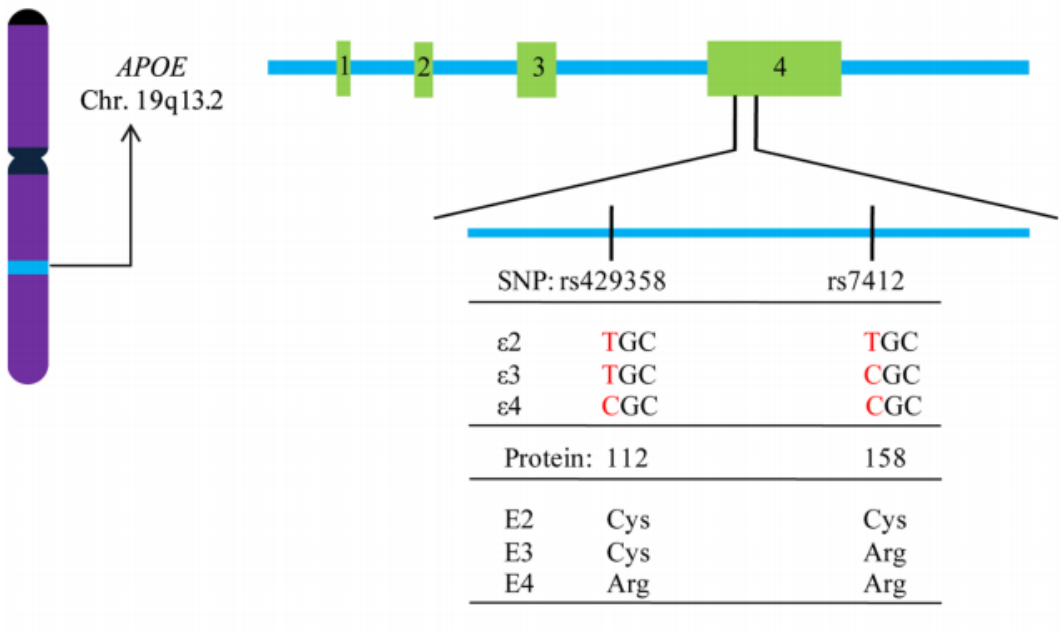


Figure 1.4: A schematic diagram of human *APOE* gene (Zhong, et al. 2016).

1.7.3 Mechanism of Action of *APOE* Gene

APOE gene encodes for apolipoprotein E which is a plasma protein, containing 298 peptide bonds that consist of four exons and three introns (Sapkota, et al. 2015), (Jiang, et al. 2017) & (Zhen, et al. 2018). The plasma glycoprotein apoE helps in clearance of lipoprotein from circulation. ApoE, in periphery, is synthesized by the liver and macrophages, while in the brain, it is produced by astrocytes. ApoE is a carrier of chylomicrons, very low density lipoproteins intermediate density lipoproteins, and high density lipoproteins. It serves as a ligand for hepatic apoE receptor or LDL receptor by binding to it to take the apoE containing lipoproteins. The binding properties to apoE

receptor differ among the different apoE isoforms. It was discovered that apoE3 and apoE4 bind to receptor having similar affinity, but apoE2 has only 2% of this affinity which leads to defects in lipoprotein metabolism that cause atherosclerosis (Zhong, et al. 2016) & (Elmadbouh, et al. 2013).

1.7.4 Studies on *APOE* Gene

Several studies have been done to study the relationship between *APOE* gene and diseases. For instance, Sapkota and others have shown in their study on Chinese population that ApoE4-isoform people are at a high risk of coronary artery disease and Alzheimer's disease by which the E2 and E3 isoforms prevent the Tau protein aggregation by limiting abnormal phosphorylation (Sapkota, et al. 2015). On the other hand, the E4 isoform cannot limit the abnormal phosphorylation. Moreover, people having high body cholesterol with apoE4 isoform have an increased risk of T2D and heart disease compared to those who have E3, but E2 carriers are considered as protective (Zhen, et al. 2018). A meta-analysis done by Yin et al. among Chinese population has shown that apoE2 and apoE4 alleles are risk factors for T2D development and diabetic nephropathy (DN) (Yin, et al. 2014). However, Zhang et al. have found in their meta-analysis that allele E2 and the genotype E2/E3 are risk factors for type 2 diabetic nephropathy but allele E3 and the genotype E3/E3 are protective and E4 has no significance (Zhang, et al. 2015). Another study showed that apoE2 and E4 were correlated with the increased risk of type 2 diabetes mellitus (Yin, et al. 2014) & (Livny, et al. 2016). However, there was no association between *APOE* gene polymorphism and diabetic nephropathy among Turkish population (Erdogan, et al. 2009). A study on Israeli population has shown that poor glycemic control is obtained in people who have apoE4 allele resulting in a high HbA1c levels (Ravona-

Springer, et al. 2014). So, different ethnic groups show different correlations of apoE isoforms with type 2 diabetes mellitus. Table 1.2 summarizes different association among different ethnic groups.

Table 1.2: Relationship between *APOE* and diseases among different ethnic groups.

The population	**Studied relationship**	**Is there association?**
Chinese population	*APOE2* , *APOE4* and risk factors for T2DM	Yes
South Asian popoulation	ApoE4 isoform and lipidemicT2D patients	Yes
Turkish population	*APOE* gene polymorphism and diabetic nephropathy	No
Japanese population	E2 isoform and lipidemic T2DM patients	Yes
Omani population	*APOE* genotype and coronary artery diseases in lipidemic population	No
Kuwaiti population	E4 allele and cardiovascular disease in T2DM pateints	Yes
Egyption population	E4 allele and risk of T2DM patients	Yes

1.7.5 *APOE* Gene Promoter

Several studies have been done to associate *APOE* promoter with human diseases. *APOE* promoter has three SNPs; 2491A/T (rs449647), 2219G/T (rs405509) and +113G/C (rs440446). Each allows *APOE* to function differently. For example, the *APOE* promoter 2491A genotype has been correlated with an increased plasma level of apoE and also increased risk for Alzheimer's disease as compared to 2491T genotype (Geng, et al. 2011). Lambert and others found out that a cis mutation in the *APOE* promoter may favor the expression level of the E4 isoform, and potentially the harmful action of the E4 allele (Lambert, et al. 1998).

1.7.6 *APOE* Gene Info

Table 1.3: Summary of *APOE* gene (https://www.ncbi.nlm.nih.gov/gene/348).

Official symbol	*ApoE* provided by HGNC
Full gene name	Apolipoprotein E provided by HGNC
Chromosomal location	19q13.2
Exon number	4 exons
Gene ID	348
Organism	Homo sapiens
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo
Gene type	Protein coding
known as	AD2; LPG; APO-E; ApoE4; LDLCQ5
Expression	Biased expression in liver (RPKM 1021.7), kidney (RPKM 648.1) and 7 other tissues

1.8 Study Objectives

In Palestine, it is not known if there is a correlation between *APOE* gene polymorphism and diabetes. The aim of our study is to investigate the relationship between *APOE* genotype and three promoter polymorphisms with lipid profiles in T2DM Palestinians. Thus, we can also compare the results with other ethnic groups.

Specific objectives:

1. To determine the prevalence of *APOE* genotypes among Palestinian T2DM patients.
2. To assess the relationship of *APOE* genotypes with lipid profiles and diabetic complications in patients with T2DM.
3. To investigate the correlation between *APOE* promoter polymorphisms (rs449647), (rs769446) and (rs405509) and lipid profiles in T2DM patients.

1.9 Literature Review : Genetic Studies on *APOE* Gene

Some studies have been done on *APOE* gene in the Arab population (Al-Yahyaee, et al. 2005; Al-Yahyaee, et al. 2007; Al-Bustan, et al. 2005; Al Majed, et al. 2011; Galal, et al. 2020). As it has been considered as a significant genetic factor contributing to different diseases in the most of populations in the world, it is important to know whether it plays this role in human beings of Arabs or not.

An Omani research that has been published on 2004 studied the distribution of *APOE* gene alleles in the Omani population. They have shown that E3 allele has the highest percentage in the population while E4 has the lowest. This agrees with the most of other communities worldwide that the SNP that produce E3 is the most preferable one in human population (Al-Yahyaee, et al. 2005). Another study in the same population has presented that E3 allele is the most to be in both lipidemic and non-lipidemic. The study showed no relationship between *APOE* genotype and development of coronary artery diseases in lipidemic Omani population (Al-Yahyaee, et al. 2007).

Another study investigated the distribution of *APOE* genotypes in Kuwaiti people. The results are as the most, E3/E3 genotype is the commonest one in Kuwaiti people with frequency of 78.42%. Overall, E3 allele carriers are more popular than other alleles carriers (Al-Bustan, et al. 2005). Al Majed et al. has proven that there is an association between E4 allele and cardiovascular disease in T2DM Kuwaiti patients (Al Majed, et al. 2011).

A recent study made on Egyptian population showed that there is an association between *APOE* gene and T2DM and obesity. By which E4/E4 genotype and E4 allele frequencies have been seen to be higher in T2DM patients increasing the risk of its role in the disease development. While E2/E2 genotype and E3 allele are most to be in obese people making this allele involved in raising the risk of being obesity (Galal, et al. 2020).

Chapter two

2. Materials and methods

2.1 Study Subjects

In this study, a total of 204 patients were recruited from Ramallah hospital during the period from January to April 2019. All of them were Palestinian T2DM patients aged >50 at the time of sampling. Among them, 96 patients were lipidemic and 108 were non-lipidemic diabetic patients. The ratio of male to female in lipidemic group is 62/34 while it is 63/45 in non- lipidemic individuals (see table 2.1 below). As it is also shown in the table, there are no significant differences in both gender and age between the two groups as P-value is 0.36 and 0.62 respectively.

T2DM patients are defined according to the WHO criteria by which fasting plasma glucose ≥126 mg/dl or being treated with anti-diabetic agents. While dyslipidemia was characterized via one of the following: total cholesterol level ≥240 mg/dL, triglyceride level ≥150 mg/dL, low-density lipoprotein cholesterol level ≥140 mg/dL, high-density lipoprotein cholesterol level <40 mg/dL, or the use of a lipid-lowering drug. All demographic and clinical data including age, sex, BMI, treatment and diabetic complications were taken from their medical records. All biochemical and blood pressure measurements were performed during the hospital admission examination.

The study was approved by Al-Quds University ethics committee 71/REC/2019 (see appendix1). Written informed consent was obtained from all subjects before participation.

Table 2.1: Study subjects numbers

Variable	**Lipidemic** **T2DM patients**	**Non-lipidemic** **T2D patients**	***P*-value**
Gender			
Male	**62**	**63**	**0.36**
Female	**34**	**45**	
Total	**96**	**108**	
Age (mean±SD)	**62.3 ± 9.86**	**63.1 ± 10.53**	**0.62**

P-value<0.05 is considered to be significant.

2.2 Methods

2.2.1 Blood sampling and DNA extraction

Approximately, 5 ml whole blood from patients after 12 hours fasting was collected in tubes containing 0.5 ml of ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant. Samples were stored at 4°C and DNA was extracted within 24 hours after collection.

Genomic DNA was extracted from blood samples using NucleoSpin® Blood kit. Extraction steps were performed following the manufacturer's instruction. Briefly, proteinase K, buffer B3 were added to blood sample separately. Incubation was done at 70°C for 10-15 min. Then, ethanol and buffers; were added to the mixture in a NucleoSpin® Tissue Column. After the last centrifugation, the DNA samples in the tubes were stored at -20°C until further analysis. For more details, see appendix2.

2.2.2 DNA Quantification

The concentration of DNA samples was measured using Nano drop 1000 and the 260/280 and 260/230 were determined. One microliter (1µl) of DNA sample was loaded in the

correct place after blanking the device with water. Ratios of 260/280 and 260/230 were recorded for DNA quality.

2.2.3 Genetic Detection of *APOE* Gene and Promoter Polymorphisms

2.2.3.1 DNA Amplification

We have designed primers to amplify two fragments of *APOE* gene (218bp & 120bp) containing two polymorphisms of *APOE* gene (rs429358T/C and rs7412C/T) (table 2.2) that determined *APOE* genotypes. Three primers were used to amplify two fragments; a fragment of *APOE* promoter (403bp) giving us the reading of two SNPs (rs449647 T/A, rs769446 C/T) (as 500NextSeq machine reads to 150bp) and another one (194bp) containing one SNP (rs405509 A/C) (table 2.3). Schematic representation for primers location and size of targeted fragments were described in figure 2.1. The targeted region of *APOE* sequence and primer design were described in appendix 3.

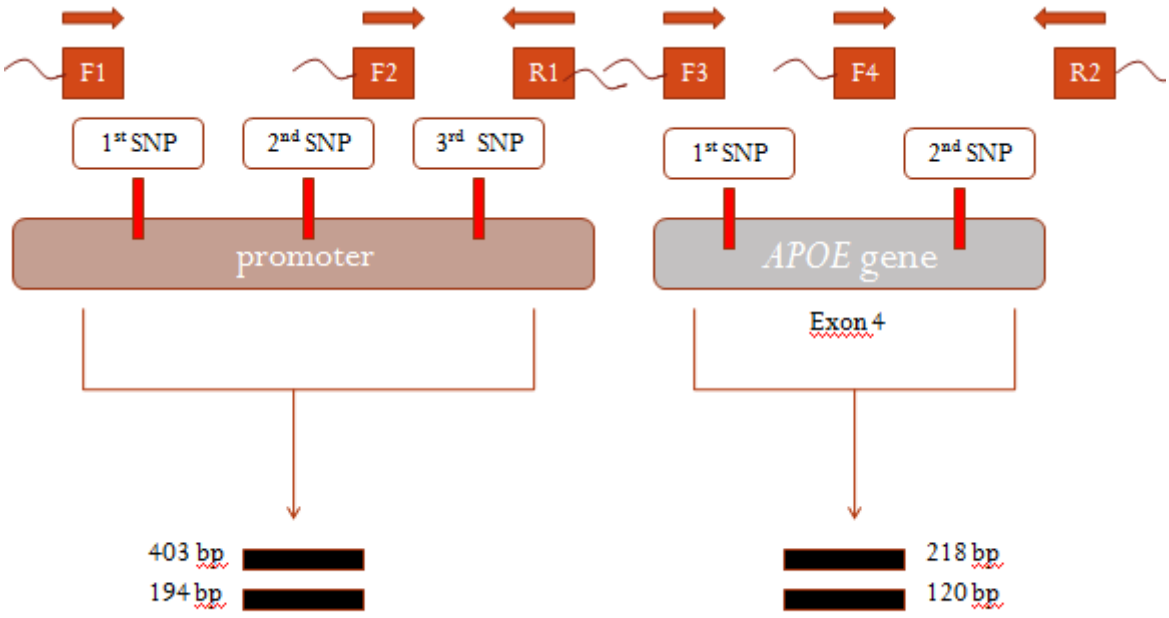


Figure 2.1: Schematic representation for primers of both APOE gene and promoter SNPs.
F1: SNPpromoterApoEF/ F2:SNP3promoterApoEF/ R1:SNP3promoterApoER/ F3:ILLULAPOEF/ F4: ILLUSAPOEF/ R2: ILLUUAPOER.
Promoter: 1st SNP: rs449647/ 2nd SNP: rs769446/ 3rd SNP: rs405509.
Gene: 1st SNP: rs429358/ 2nd SNP: rs7412.

Primers were modified with over hanged Illumina adaptor sequences at the 5′ ends in order to attach the PCR product to Illumina flow cell for deep sequencing (see tables 2.2 & 2.3).

Table 2.2: Primers used in the amplification of *APOE* gene SNPs (rs429358T/C and rs7412C/T).

PCR-Ge	Primer sequence
ILLULAPOEF (F3)	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCAAG GAGCTGCAGGCGGCGCA3'
ILLUUAPOER (R2)	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCCC GGCCTGGTACACTGCCA3'
ILLUSAPOEF (F4)	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGAGC ACCGAGGAGCTG3'

The shaded sequences are referred to adapters.

Table2.3: Primers used in the amplification of *APOE* promoter SNPs (rs449647 T/A, rs769446 C/T and rs405509 A/C).

PCR-Pr	Primer sequence
SNPpromoterApoEF (F1)	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAC GCCTGGCTAACTTTTGT3'
SNP3promapoeF (F2)	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAG GACAGGGTCAGGAAAGG3'
SNP3promapoeR (R1)	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAG GTGGGGCATAGAGGTCTT3'

The shaded sequences are referred to adapters.

The amplification of *APOE* gene SNPs was done using multiplex PCR (designed as PCR-Gem, using three primers; ILLULAPOEF,ILLUUAPOER, ILLUSAPOEF) as follow: the mixture for the PCR was prepared by adding 0.25µl of each of the three primers (10 pmol/µl), 12.5µl X2 ready mix (REDTaq®, Sigma-Aldrich Co. California 94404, USA), 7µl of distilled water and 5µl of the extracted DNA (20-50 ng). The reaction was done in total volume of 25µl. For *APOE* promoter amplification; a multiplex PCR was done using three primers with volume of 0.25µl for each. However, when sequenced, the samples showed no reads for the larger band 403. Thus, we repeated the PCR of this fragment which contains the SNPs rs449647 and rs769446 using another forward primer (see appendix 4) to amplify a region of 146bp size. Both PCR reactions started with initial denaturation at 95◦C for 5min, followed by 35 cycles; denaturation for 30sec at 95◦C, annealing for 30sec at 64◦C and elongation for 40sec at 72◦C. The last cycle has an additional step with 5min at 72◦C to assure the complete extension of PCR products. The amplification was done using Gene Amp, PCR system 9700. PCR products were stored at -20◦C until further analysis. Negative controls (PCR mix with no DNA template) were included in all amplification reactions. The PCR products were visualized and captured on a 2% agarose gel.

2.2.3.2 Gel Electrophoresis

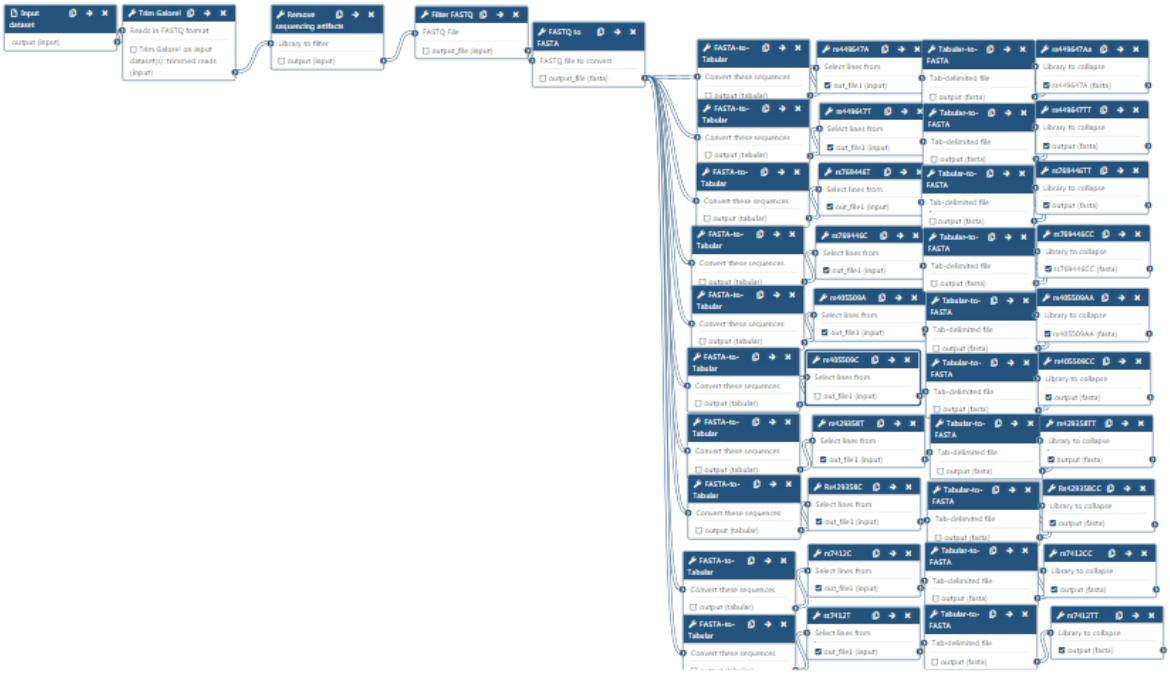
The gel was prepared by addition of 2g agarose to 100ml TAE buffer, boiling the mixture and adding 2µl(10ug/ml) ethidium bromide. The mixture is then poured into agarose gel casting system (Bio-Rad, SUB-CELL®GT). After that, solidified gel was placed into electrophoresis chamber and covered by TAE buffer and 5µl of PCR products were loaded into the wells. Finally, samples were electrophoresed /run at voltage of 120 volts for 30 min. The PCR product bands were seen using gel documentation system(GelDoc). For more details, see appendix 5.

2.2.3.3 Genotyping by Amplicon based Next Generation Sequencing (NGS)

The PCR product (20µl) of the PCR-Ge (epsilon) and 20µl of PCR-Pr (promoter) of each individual sample were mixed together in a tube with a volume of 40 µl, cleaned by AgencourtAMPure XP system (X1, A63881; Beckman Coulter Genomics, Indianapolis, IN, USA) as follow: starting with the addition of magnetic beads that are carboxy coated to DNA sample and vortexed. The mixture was set at room temperature for five minutes to give the nucleic acids time to bind to the surface beads. Then, the mixture was put in a magnetic rack resulting in the move of beads bound to nucleic acids to the back of the tube attracted to the magnet. When all the beads had been out of the solution, the solution became clear. After that, the nucleic acid was stuck to the beads, so we removed the supernatant. Washing was done twice by adding 80% ethanol carefully. Then, samples were dried from any ethanol residuals. And eluted in 25ul elution buffer. All purified products were subjected to a second round of amplification to assign unique index sequences (barcode) for each sample using Nextera XT Index Kit (Illumina, San Diego, CA, USA). 5µl from each barcoded sample were pooled together, mixed and spin down. Then, 100 µl of the pooled product was cleaned by AgencourtAMPure XP system (X1) (A63881; Beckman Coulter Genomics), and eluted in 50 µl elution buffer. Library purity and quantity were evaluated by QubitFluorometer (Invitrogen, Carlsbad, CA, USA) using QubitdsDNA high-sensitivity assay (Invitrogen, Carlsbad, CA, USA). A concentration of 4 nM was prepared. Samples were sent for hylab company for sequencing, 20 K reads for each sample were targeted. The company used IlluminaNextSeq 500/550 machine, and 150-cycle Mid Output Kit (Illumina, San Diego, CA, USA) for deep sequencing. The obtained DNA sequences as fastq files were uploaded on the Galaxy program (https://usegalaxy. org/). Workflow of filtration included Illumina adaptor trim, quality

selection of Q > 20 with minimal read length of 100bp, the workflow is shown in figure 1.2.

Figure 2.2: The workflow.



Ten virtual probe sequences; four for the gene (table 2.4) and six for the promoter (table 2.5) were used to identify polymorphisms. The genotypes were determined based on the calculated ratio between the read counts for wild type and mutant alleles, for all SNPs (*APOE* gene and promoter) in each individual sample.

Table 2.4: The virtual probes used to target *APOE* gene SNPs (rs429358T/C and rs7412C/T).

rs429358T	GACGTGTGCGGC
rs429358C	GACGTGCGCGGC
rs7412C	GCAGAAGCGCCTGG
rs7412T	GCAGAAGTGCCTGG

The shaded nucleotides are the ones producing SNPs.

Table 2.5: The virtual probes used to target *APOE* promoter SNPs (rs449647 T/A, rs769446 C/T and rs405509 A/C).

rs449647A	TCTCAAACTCCTG
rs449647T	TCTCAATCTCCTG
rs769446T	GTGAGCTACCGC
rs769446C	GTGAGCCACCGC
rs405509A	GTCTGAATTACTG
rs405509C	GTCTGCATTACTG

The shaded nucleotides are the ones producing SNPs.

2.2.3.4 Statistical Analysis

The results were analyzed using SPSS version 23. T-test, Fischer Exact and Chi-square ($\chi 2$) tests were applied to the results as they can assess for independence between two variables when the groups we are comparing are not correlated and independent. It is common that Fischer exact test is used for small sample size or when frequencies are less than 5, while Chi-square used for large sample size (Kim, 2017). Odd ratio (OR) was also measured as it is used to compare the relative odds of the outcome occurrence according to the variable. Through it, we can determine if the exposure to a variable increases the risk of the outcome or not. When OR equals 1, it means that there is no association; OR>1, exposure correlated with higher odds of outcome; OR <1, exposure correlated with lower odds of outcome (Szumilas, 2010). These were done to compare between lipidemic and non-lipidemic diabetic patients according to their genotypes.

Chapter Three

3. Results

3.1. Clinical and Biochemical Characteristics of the Study Subjects

The table below (table 3.1) shows a comparison of clinical and biochemical characteristics between lipidemic and non-lipidemic diabetic participants. The results represent a significant difference in triglycerides between the two groups as *P*-value is $4.18*10^{-9}$ which is less than 0.05. Moreover, it has been shown that total cholesterol is significantly higher in lipidemic T2DM with *P*-value of $5.34*10^{-12}$. There is also a significant difference between lipidemic and non-lipidemic T2DM in HDL as it presents in larger amounts in non-lipidemic patients (P-value=$3.94*10^{-9}$).

LDL is seen to be significantly different between the two groups as lipidemic has higher LDL level, and P-value is $1.06*10^{-17}$. While SBP,DBP, HbA1c and FBS showed no significant differences between lipidemic and non-lipidemic as P-value>0.05.

Table 3.1: Clinical and biochemical characteristics differences between lipidemic and non-lipidemic T2D patients.

Variable	**Lipidemic T2D patients**	**Non-lipidemic T2D patients**	***P*-value**
SBP	135.02±18.75	139.85±20.49	0.08
DBP	77.79±13.11	81.06±11.15	0.07
HbA1C	8.02±1.3	8.13±1.5	0.59
FBS	241.62±108.66	227.22±92.82	0.31
TG	252.6±148.79	149.68±60.32	$4.18*10^{-9}$
TC	327.38±86.47	161.78±50.7	$5.34*10^{-12}$
HDL-C	36.07±13.17	49.58±17.99	$3.94*10^{-9}$
LDL-C	161.51±54.32	96.18±41.64	$1.06*10^{-17}$

P-value<0.05 is considered to be significant.

3.2 Genotyping

3.2.1 DNA Concentration and Purity Measurement

Nanodrop 1000 was used to measure the concentration and purity of DNA in samples. The DNA concentration was in ng/µl while the purity was estimated by 260/280 and 260/230 ratios.

The concentration of DNA should be more than 1ng to be eligible for PCR machine. The concentrations of our samples were in the range of 50-200ng/µl which indicates good DNA extraction.

Table 3.2 represents concentration and purity of six samples.

260/230 Ratio

260/230 ratio is to indicate purity of our DNA samples. The normal ratio should be in the range of 2.0-2.2. Samples with contaminants such as phenol, which absorb light at 230 nm wave length, will not show a ratio near that one and this means that the DNA extraction protocol was not well-performed.

260/280 Ratio

Proteins are detected at 280nm absorbance. A ratio of ~ 1.8 is considered to be accepted as pure DNA. Abnormal ratio shows a contamination with proteins. This problem could be detected by the addition of proteinase K which plays its role in degrading the proteins and therefore eliminated from the sample. The table below shows that sample number 4 with 56.7ng/µl has a 260/280 value of 1.84 which is very close to 1.8. And its 260/230 ratio is 1.69.

Table 3.2: Concentration and purity of DNA in representative samples (n=6).

DNA sample	ng/µl	260/280	260/230
1	59.39	1.97	1.08
2	56.7	1.84	1.69
3	110.84	2.85	1.99
4	60.37	2.95	1.78
5	114.03	2.04	1.99
6	98.54	1.82	1.61

3.2.2. Amplification of DNA

PCR of *APOE* gene

As expected, the PCR product of *APOE* gene revealed two bands at the level of 218bp and 120bp respectively targeting the two polymorphisms (rs429358 T/C and rs7412 T/C). The figure below (3.1) shows the gel electrophoresis that was done to see the PCR products.

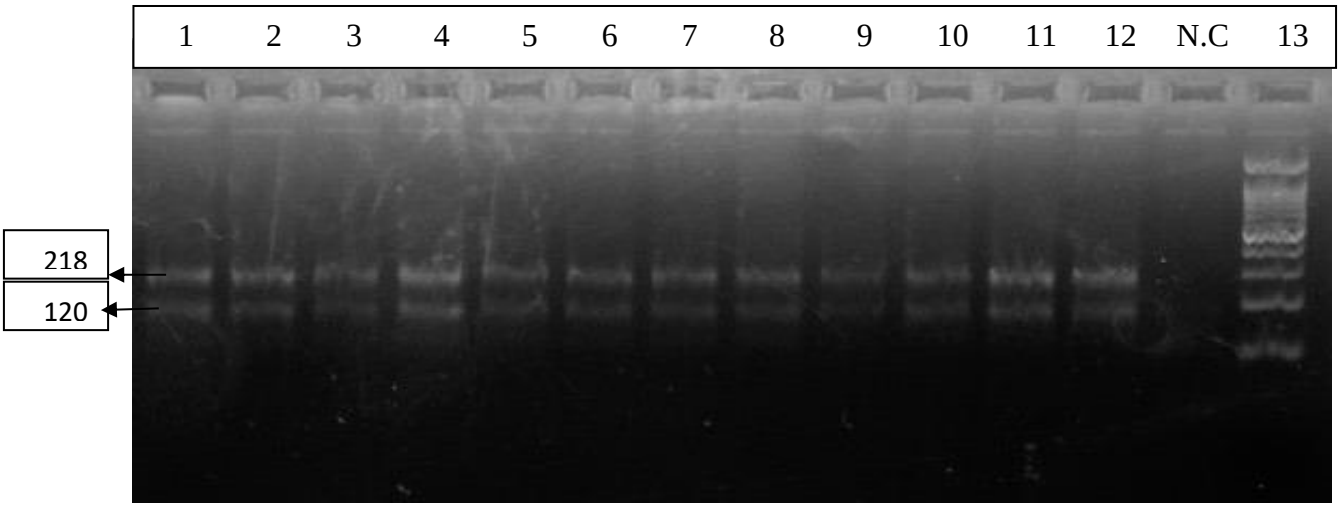


Figure 3.1: PCR products of *APOE* gene polymorphisms (rs429358 T/C and rs7412 T/C) analyzed by agarose gel electrophoresis.
Line13 refers to DNA ladder (50 bp); N.C: negative control; 1-12 PCR products (sizes of amplicons 218 and 116).

PCR of *APOE* promoter

The *APOE* promoter amplification produces two bands with sizes of 403bp giving us reads for two SNPs (rs449647 & rs769446) and 194bp (containing one polymorphism (rs405509)). Figure 3.2 below shows the bands that appeared in the gel electrophoresis.

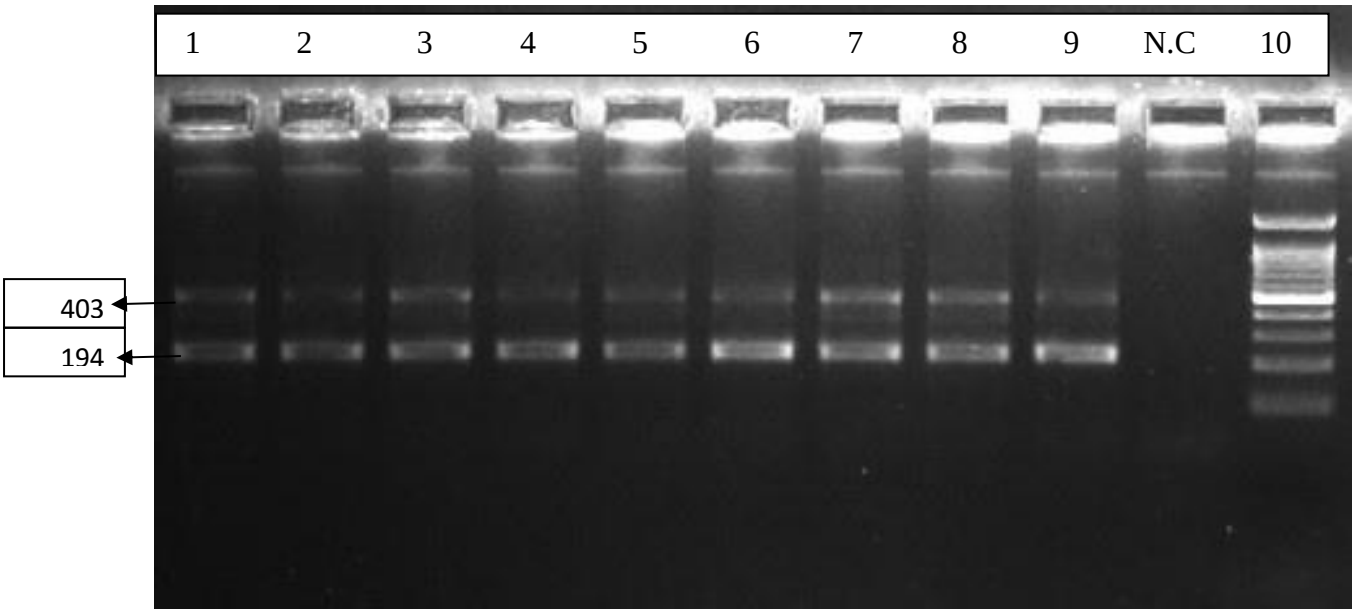


Figure 3.2: PCR products of *APOE* promoter polymorphisms (rs449647, rs769446 and rs405509) analyzed by agarose gel electrophoresis.
Line10 refers to DNA ladder (50bp); N.C: negative control; 1-9 PCR products (sizes of amplicons403 and 194).

3.2.3 Sequencing of *APOE* Gene and Promoter Polymorphisms

The genotypes for *APOE* gene and promoter SNPs were determined by amplicon based next generation sequencing. The three allelic variants of *APOE* (*APOE* epsilon polymorphisms) were defined by two SNPs, (rs429358 and rs7412) combination to ε2, ε3 and ε4 based on data in https://www.snpedia.com/index.php/APOE, (table 3.3).

Table 3.3: The allelic name according to *APOE* gene SNPs

rs429358	Rs7412	Name
C	T	Ɛ1
T	T	Ɛ2
T	C	Ɛ3
C	C	Ɛ4

Table 3.4: *APOE* genotype and alleles frequency in lipidemic (96) versus non-lipidemic T2DM (108) patients.

Genotype/allele	**All subjects**	**Lipidemic T2D patients "n" (%)**	**Non-lipidemic T2D patients "n" (%)**	***P*-value**
E2/E2	5	4 (4.2)	1 (0.9)	0.19
E2/E3	19	8 (8.3)	11 (10.2)	0.81
E2/E4	3	2 (2.1)	1 (0.9)	0.60
E3/E3	162	75 (78.1)	87 (80.6)	0.73
E3/E4	15	7 (7.3)	8 (7.4)	1
E2 allele	27	14 (12.4)	13 (10.2)	0.68
E3 allele	196	90 (79.6)	106 (82.8)	0.62
E4 allele	18	9 (8)	9 (7)	0.81

*Fisher Exact Test, P-value <0.05 is considered to be significant.

E2/E2 is defined by the presence of two *APOE* alleles carrying T in both SNPs.

E2/E3 is defines by the presence of one *APOE* allele carrying T in both SNPs and the other allele carries T in one SNP rs429358 and C in the other SNP Rs7412.

E2/E4 is defined by two *APOE* alleles, one of them carries T in both SNPs, the other carries C in both SNPs.

E3/E3 is defined by the presence of two *APOE* alleles and both carries T in SNP rs429358 and C in the SNP Rs7412.

E3/E4 is defined by *APOE* allele carrying T in one SNP rs429358 and C in the other SNP Rs7412 and the other allele carries C in both SNPs.

Table 3.5: Genotyping and allelic frequency of 3 SNPs in the *APOE* promoter region among lipidemic versus non-lipidemic T2DM patients.

SNP rs	**genotype/allele**	**Lipidemic n (%)**	**Non-lipidemic n (%)**	**P-value**
rs769446	TT	87 (91)	94 (87)	0.14
	TC	9 (9)	14 (13)	
	CC	0 (0)	0 (0)	
	T	183 (95)	202 (94)	0.14
	C	9 (5)	14 (6)	
rs449647	AA	42 (43.75)	42 (39)	0.07
	AT	41 (42.7)	51 (47)	
	TT	13 (13.55)	15 (14)	
	A	125 (65)	135 (62)	0.28
	T	67 (35)	81 (38)	
rs405509	GG	26 (27)	31 (29)	0.58
	GT	48 (50)	51 (47)	
	TT	22 (23)	26 (24)	
	G	100 (52)	113 (52)	0.44
	T	92 (48)	103 (48)	

*Adjusted for age, gender and BMI, P-value<0.05 is considered to be significant.

As shown in tables 3.4 and 3.5, there were no significant differences in the genotype and allele frequency in both *APOE* gene and *APOE* promoter SNPs (P-values>0.05) among the lipidemic and non-lipidemic T2DM patients.

3.2.4 Association of *APOE* Gene and *APOE* Promoter SNPs with Risk of Dyslipidemia

The association of APOE genotype and the risk of dyslipidemia was estimated by logistic regression analysis adjusted by age, gender and BMI. There were no significant differences between lipidemic and non-lipidemic T2DM patients according to genotypes of *APOE*.

The E3/E3 genotype was considered as reference genotype as shown in table 3.6.

Table 3.6: Association of APOE gene polymorphisms with risk of dyslipidemia.

Genotype	**case (n)%**	**control (%)**	**adj OR 95%CI***	***P* value**
E2/E2	4 (4.2)	1 (0.9)	0.28 (0.01-3.66)	0.36
E2/E3	8 (8.3)	11 (10.2)	1.25 (0.39-4.17)	0.71
E2/E4	2 (2.1)	1 (0.9)	1.34 (0.05-20.20)	0.83
E3/E3	75 (78.1)	87 (80.6)	1	NA
E3/E4	7 (7.3)	8 (7.4)	0.67 (0.17-2.67)	0.57

*Adjusted for age, gender and BMI. P-value<0.05 is considered to be significant.

Table 3.7: Association of *APOE* promoter SNPs with risk of dyslipidemia.

SNP rs	genotype/allele	Case n (%)	Control n (%)	OR*	95% CI*	P-value
rs769446	TT	87 (91)	94 (87)	1.00	NA	0.14
	TC	9 (9)	14 (13)	0.44	(0.14-1.34)	
	CC	0 (0)	0 (0)	NA	NA	
	T	183 (95)	202 (94)	1.00	NA	0.14
	C	9 (5)	14 (6)	0.44	(0.14-1.34)	
rs449647	AA	42 (43.75)	42 (39)	1.00		0.07
	AT	41 (42.7)	51 (47)	0.42	(0.19-0.92)	
	TT	13 (13.55)	15 (14)	0.81	(0.29-2.30)	
	A	125 (65)	135 (62)			0.28
	T	67 (35)	81 (38)	0.76	(0.46-1.25)	
rs405509	GG	26 (27)	31 (29)	1.00	NA	0.58
	GT	48 (50)	51 (47)	0.96	(0.42-2.19)	
	TT	22 (23)	26 (24)	1.51	(0.56-4.09)	
	G	100 (52)	113 (52)	1.00	NA	0.44
	T	92 (48)	103 (48)	1.22	(0.74-2.00)	

*Adjusted for age, gender and BMI, P-value<0.05 is considered to be significant.

Further, there were no significant differences between lipidemic and non-lipidemic T2DM patients according to APOE promoter SNPs as p-values were higher than 0.05. See table 3.7.

3.2.5 Comparison of Lipid Profile among Subjects Carrying E3/E3 Genotype, E4 and E2 Carriers

The study population was stratified into three sub groups, according to genotypes, the first group included all individuals who have the most common genotype (E3/E3), the second group included all patients who have at least one E4 allele and the third group included all patients who have at least one E2 allele. T-test was done to compare lipid profiles between these three sub groups across the lipidemic and non-lipidemic T2DM patients. As shown in table 3.8, the stratified analyses revealed no evidence for association between genotypes and lipid levels (TC, TG, HDL, LDL) (P>0.05).

Table 3.8: Comparison of lipid profile among subjects carrying E3/E3 genotype, E4 and E2 carriers.

Variable			**Lipidemic T2D patients**				**Non-lipidemic T2D patients**			
	E3/E3	**E4**	***P* value***	**E2**	**P value**	**E3/E3**	**E4**	***P* value***	**E2**	**P value**
TC	238.37 ± 91	232.44 ±77.95	0.84	234 ±63	0.84	160.78 ±49.19	162.78 ±66.16	0.93	168 ±51	0.66
TG	251.53 ±161.56	251.56 ±116.82	1	247 ±77	0.84	150.15 ±62.02	131.67 ±37.19	0.21	156 ±62	0.75
HDL	36.57 ±13.93	36.44 ±8.38	0.97	33 ±10	0.26	49.01 ±18.42	48.33 ±14.44	0.90	56 ±17	0.18
LDL	164.23 ±56.55		0.12	158 ±43	0.65	90.89 ±35.17	114.44 ±68.68	0.34	117 ±51	0.09

Data presented as mean+SD

E4=E2/E4+E3/E4

E2= E2/E2+E2/E3+E2/E4

*t-test statistic

3.2.6 Genotype Frequencies of the Three Promoter SNPs According to *APOE* Genotypes

To examine whether the frequency of *APOE* promoter genotypes may differ across the *APOE* genotypes, the study population were stratified into three groups E2, E4 and E3/E3 carriers. No significant differences in the frequency of *APOE* promoter SNPs across the *APOE* epsilon genotype (table 3.9). Genotype frequencies of rs769446 according to the *APOE* status showed that in E2 subjects the frequency of T/T genotype was lower in non lipidemic than in lipidemic (12 vs 13). There was found a significant difference between lipidemic and non-lipidemic in TC genotypes in E2 carriers as OR=0.01 and P-value< 0.05. And no C/C genotypes were found in both groups. Similarly, genotype frequencies of rs449647 according to the APOE status are reported in table 3.9 and showed that in E4 subjects the frequency of A/A genotype was lower in lipidemic than in non lipidemic. It was also found that in E4 carriers there is a significant difference between the two groups in both AT and TT as OR=0 and P-value<0.05. However the sample size is small so this result should be confirmed.

Table 3.9: Genotype frequencies of the promoter SNPs according to *APOE* genotypes.

APOE* genotypes	**promoter SNPs**	**lipidemic (n)**	**non lipdemic (n)**	**OR (95% CI)****	**P-value**
	rs769446				
E2 carriers	TT	13	12	1	0.04
	TC	1	1	0.01 (0.00-1.37)	
	CC	0	0	NA	
E3/E3 carriers	TT	68	74	1	0.28
	TC	7	13	0.53 (0.17-1.70)	
	CC	0	0	NA	

E4 carriers	TT	7	9	1	0.86
	TC	2	0	NA (0.00-NA)	
	CC	0	0	NA	
	rs449647				
E2carriers	AA	5	4	1	0.14
	AT	8	4	0.12 (0.00-7.46)	
	TT	1	5	0.03 (0.00-1.66)	
E3/E3 carriers	AA	35	34	1	0.15
	AT	31	43	0.47 (0.21-1.09)	
	TT	9	10	1.09 (0.32-3.64)	
E4 carriers	AA	2	4	1	0.02
	AT	4	4	0.00 (0.00-NA)	
	TT	3	1	0.00 (0.00-NA)	
	rs405509				
E2 carriers	GG	2	4	0.27 (0.01-5.90)	0.16
	GT	8	4	7.95 (0.24-264.25)	
	TT	4	5	1	
E3/E3 carriers	GG	22	25	1	0.44
	GT	36	42	0.78 (0.31-1.93)	
	TT	17	20	1.46 (0.48-4.41)	
E4 carriers	GG	2	3	1	0.53
	GT	5	5	23.80 (0.01-NA)	
	TT	2	1	9.39 (0.00-NA)	

*Adjusted for age, gender and BMI. P-value<0.05 is considered to be significant.

E2=E2/E2+E2/E3+E2/E4.

E4=E2/E4+E3/E4.

Chapter Four

4.1 Discussion

Type 2 Diabetes mellitus is considered as one of the most common metabolic diseases worldwide. It is a 10 top cause of mortality killing 1.6 million people in the world (Oguntibeju, et al. 2019). People with diabetes are not able to make or use insulin and thus glucose does not enter the cells and remain outside elevating its level in the blood. This affects the whole body and could lead to real complications if it remained uncontrolled. Complications could be microvascular or macrovascular, the first includes blindness, kidney failure, and etc. Examples of the latter are stroke related deaths, cardiovascular diseases (CVD), …etc. Studies show that stroke related deaths are 2-4 times more common in diabetic patients than in unaffected people. Type 2 diabetes is a multifactorial disease caused by both genetic and non-genetic factors such as energy intake and life style related factors (i.e. exercise and smoking,…). Changes in the diabetes prevalence in various populations with different ethnicity prove this relationship. Understanding of diabetes genetics is essential to the development of new methods of treatment and strategies of effective prevention of this disease. Apolipoprotein E (*APOE*) gene polymorphism has been implicated in predisposition to diabetes and dementia in old population. This gene codes for a protein responsible of providing the main core of lipoproteins elements by which it maintains the lipid homeostasis in both blood and periphery (Zhong, et al. 2016), it also has a vital function in lipoprotein transportation between tissues and body fluids (interstitial fluid and plasma). Regulation of metabolism of cholesterol and triglycerides is conducted by the role of this gene product (Da Wei, et al. 2019).

The relationship between *APOE* gene polymorphism and T2DM has been studied well worldwide. Several researchers have found significant associations between *APOE* polymorphisms and T2DM patients. As examples, a meta-analysis study on Chinese

population has been conducted in 2017 and showed that carriers of E2 allele have elevated risk to T2DM, while E2/E3 genotype increased the possibility of being affected with diabetic nephropathy. On the other hand, E4 allele was found to be a leading factor to the progress of T2DM and severe diabetic peripheral neuropathy. Moreover, E4 allele was significantly associated with mortalities in T2DM patients (Zhang, et al. 2017). Another study on Chinese population was published in 2020 presented that *APOE* is associated with diabetic nephropathy (DN). They showed that E3 and E4 have similar binding affinity to LDL-receptor, while E2 has lower affinity. The leading factor to this is that in E2, cysteine to arginine substitution at position 158 forms salt bridge between Arg150 and Asp 154 causing weak affinity to bind to LDL-receptor (Shi, et al. 2020).While in Turkish population, there was no significant association between *APOE* gene polymorphism and Diabetic Nephropathy (DN) (Erdogan, et al. 2009). Different results are obtained among different populations and this is due to variety in ethnicity and environment.

To our knowledge, this is the first study that evaluates the association of *APOE* gene polymorphisms with the risk of dyslipidemia among T2DM patients in Palestine. Our study is the first in Palestine to describe the use of NGS in *APOE* genotyping, the method proved to be cost-effective and efficient using large number of samples and more accurate than the traditional restriction fragment length polymorphism (RFLP) and Sanger sequencing studies. In this research, five polymorphisms were studied, two of them in exon 4 of *APOE* gene, the combination of these polymorphism giving the *APOE* epsilon genotype classification. The other three polymorphisms were in the promoter region. Our results revealed that the frequency of E3 allele was the highest (79.6%) among other alleles; E3/E3 genotype was also found to be highest in all T2DM subjects with a frequency of 78.1%. Similar results were reported from a study done on Egyptians showed a frequency of 58% for E3/E3 genotype and 67.5% for E3 allele (Galal, et al. 2020), also in

Omani population E3/E3 genotype frequency was 89.4% (Al-Yahyaee, et al. 2007), 92.5 % in Jordanian (Khabour and Abdelhalim, 2020), 88.1% in India (Singh, et al. 2006), and 90 % in Italian (Gurinovich, et al. 2019). E3 allele and E3/E3genotype are considered to be the most common among all populations (Margeta, et al. 2020).

The other three polymorphisms that we studied were in the promoter region. The promoter elements are located within the region spanning - 360 to - 80 within the first intron (Laws, et al. 2003). These polymorphisms (rs405509, rs769446, and rs449647) were also known as the −219G/T, −427T/ C, and −491A/T polymorphisms respectively, and reported to be associated with plasma apoE concentration and risk of coronary heart disease (CHD) (Mannila, et al. 2013). Another research showed that there is an association between the *APOE* promoter SNP rs769446 and Alzheimer's disease independently from the confirmed correlation between E4 allele and Alzheimer's disease (Artiga, et al. 1998), (Bekris, et al. 2010) & (Fritsche, et al. 2009).

Most of association studies are done on diabetic and non-diabetic patients, as dyslipidemia is considered a risk factors for the macrovascular complications of diabetes, here we thought that the lipidimic person could have distinct *APOE* genotype and different from that of non-lipidemic. Therefore, the study population was stratified according to dyslipidemia, the obtained results showed no statistical differences (P>0.05) between *APOE* genotypes frequency (*APOE* epsilon polymorphisms and promoter SNPs) among lipidemic and non lipidemic groups.

4.1.1 Association between *APOE* (epsilon and promoter polymorphisms) and Lipid Status of T2DM Patients

In this study, Logistic regression analysis adjusted for age, gender and BMI showed no association between *APOE* genotypes (epsilon and promoter polymorphisms) and risk of

dyslipidemia. In this regard several studies showed different results: a study done on Japanese population, suggested that the presence of E2 allele may have a greater impact on higher level of plasma TG and cholesterol levels as observed in E2 carriers of T2DM patients (Eto, et al. 2002). A study held on Italian people showed the great role of apoE lipoproteins containing HDL upregulate ATP binding cassette transporter A1 expression and thus increases the efflux of Cholesterol (Palombo and Kozakova, 2020). Further, serum apoE concentrations were previously demonstrated to be elevated in patients with T2DM. Thus, the relationship is different among different ethnic groups. To infer, the degree of E2 effect on remnant lipoprotein metabolism may be different among different ethnic groups due to dietary habits differences and other genetic factors that are involved in lipid metabolism (Eto, et al. 2002). In this study, to evaluate the possible synergistic effect between the promoter SNPs and the common *APOE* polymorphism, we therefore classified the promoter polymorphisms according to the E2 ,E3/E3 and E4 *APOE* genotypes. Different studies have explained the role of the promoter polymorphisms in modifying the activity of transcription and expression of *APOE* coding region (Bizzarro, et al. 2009). Rs405509 (−219 T/G) TT is known to be correlated with reduced apoE levels than those with GG or GT in a study that has done on Korean people, and this is therefore increasing the lipid levels as there are decreased level of lipoproteins that clear them (Choi, et al. 2019). Moreover, rs405509 has been shown to control the expression of the *APOE* gene, and modifies the effect of APOE E4 on the susceptibility of AD. Rs405509-T has only 60% promoter activity in comparison with rs405509-G (Huang, et al. 2020). In this study we found no significant differences between lipidemic and non lipidemic groups after stratification of the promoter SNPs with the *APOE* genotypes. Odd ratios were less or more than 1 which means that there are differences but P-values were higher than 0.05 making these differences to be insignificant. Among E4 carriers, rs449647-T was more

frequent in lipidemic group. Moreover, in E2 carriers of both lipidemic and non-lipidemic, a significant difference has been obtained between them according to TC (rs769446) with an OR=0.00<1 and P-value<0.05. However, due to the small sample size we were unable to draw a precise conclusion using sub group analysis.

4.1.2 Association between *APOE* Genotypes (epsilon polymorphisms) and Lipid Profile among Lipidemic and Non Lipidemic T2DM Patients

In the current study, notable difference of serum cholesterol, TG and HDL-C and LDL levels were observed among participants with and without dyslipidemia ($P < 0.05$). The difference in serum lipid levels observed among the two groups indicating the impaired lipids metabolism in lipidemic T2DM subjects. As *APOE* is a candidate gene for the development of T2DM due to its critical role in the lipid metabolism, our study suggested that lipid profile could differ according to *APOE* genotype in lipidemic and non-lipidemic T2DM participants. Furthermore, a study conducted by Chaudhary and colleagues, reported that E4 allele-diabetic carriers showed a significantly higher serum TG and lower HDL-C levels compared to E3/E3 genotype carriers (Chaudhary, et al. 2012). Other studies have shown that E3 and E4 carriers have higher LDL and TC levels than E2 (Atageldiyeva, et al. 2019). Abnormal LDL receptor affinity increases its regulation, resulting in increased removal of LDL in patients carrying E2 allele (Weber, et al. 2016). It is reported that E4 is associated with increased lipids level as a result of increased absorption of cholesterol (Dan, et al. 2010). Therefore and in attempts to explore such association, we compared the lipid profile according to *AOPE* genotypes (E4 vs E3/E3 vs E2) among the two groups (lipidemic and non lipidemic T2DM patients). Our results revealed that P-values were more than 0.05 showing that there are no differences between lipid profile and *APOE* genotypes among the studied groups. In agreement to our results the lipid profiles were not correlated with *APOE* polymorphisms in Tunisian population (Chaaba, et al. 2008).

Inconsistency with our results, Dankner and others found that E2 allele has lower binding affinity to LDL receptor than E3 and E4 leading to lower cholesterol level (Dankner, et al. 2020). Another study also showed the total cholesterol was lower in E2 carriers (Zhou, et al. 2020). In addition, Goldberg et al. had obtained similar results that E2 carriers have less TC and LDL levels (Goldberg, et al. 2020). This provides that E2 allele is protective against hypercholesterolemia (Wong, et al. 2019). Some limitations of the present study should bead dressed; firstly, the small sample sizes a major drawback of the current study.

Secondly, the serum lipid profile was reported to be influenced by dietary fat-containing foods intakes. Thus, the comparison of dietary intakes of fat-containing foods between the 2 groups is needed. Thirdly, a defect in lipid metabolism can't be attributed to a single gene such as *APOE* more genetic factors should be studied. In the future, long time cohort studies needed to investigate how genetic background modulates the association between diabetes and dyslipidemia. On the other hand, epigenetic mechanism, such as DNA methylation, can regulate gene expression while the DNA sequence remains the same. Several studies have suggested that DNA methylation (at cytosines of CpG dinucleotides), is associated in pathogenesis of many human diseases, (i,e. cancers and atherosclerosis). It is reported that methylation of the *APOE* promoter could modify gene expression and thus influence blood lipid levels independent of *APOE* genotype (Karlsson, et al. 2018). We believe that the relationship between *APOE* promoter methylation and dyslipidemia deserves further exploration and research.

4.2 Conclusions

To conclude, E3/E3 was the dominant genotype in the Palestinian population. There was no association between *APOE* genotype and lipid status of T2DM. Moreover, no

relationship has seen between *APOE* promoter SNPs (rs449647, rs769446 and rs405509) and T2DM patients neither lipidemic nor non-lipidemic in Palestinian population.

4.3 Recommendations

1- A case-control study should be done between diabetic and non-diabetic people in accordance of *APOE* gene to assure the relationship between *APOE* genotype and T2DM in Palestine.

2- Larger sample size should be included in another similar study to emphasize whether *APOE* gene and promoter polymorphisms have a role in the lipid status of T2DM patients.

3-The association between peripheral blood leukocyte methylation levels in the *APOE* promoter region and dyslipidemia should be investigated

References

Abuhendi, N., et al. (2019). "Genetic polymorphisms associated with type 2 diabetes in the Arab world: A systematic review and meta-analysis." Diabetes research and clinical practice **151**: 198-208.

Al-Bustan, S. A., et al. (2005). "Apolipoprotein E genotyping among the healthy Kuwaiti population." Human biology: 487-498.

Al-Yahyaee, S. A. S., et al. (2005). "Distribution of apolipoprotein E alleles in the Omani population." Medical Principles and Practice **14**(2): 73-78.

Al-Yahyaee, S. A. S., et al. (2007). "Apolipoprotein E polymorphism in omani dyslipidemic patients with and without coronary artery disease." Human biology: 93-102.

AL Majed, H. T., et al. (2011). "Association between apolipoprotein E-polymorphism and Ischemic heart disease patients with or without type 2 diabetes mellitus: a preliminary study in Kuwait." Archives of Iranian Medicine **14**(6): 385-388.

Ali, O. (2013). "Genetics of type 2 diabetes." World journal of diabetes **4**(4): 114.

Amreen, S., et al. (2018). "Use of glycosylated HbA1c and random blood sugar as a screening tool for gestational diabetes mellitus in first trimester." International Journal of Reproduction, Contraception, Obstetrics and Gynecology **7**(2): 524-528.

Artiga, M., et al. (1998). "Risk for Alzheimer's disease correlates with transcriptional activity of the APOE gene." Human molecular genetics **7**(12): 1887-1892.

Atageldiyeva, K. K., et al. (2019). "Apolipoprotein E genetic polymorphism influence the susceptibility to nephropathy in type 2 diabetes patients." Gene **715**: 144011.

Aydin, Ö., et al. (2018). "The gut microbiome as a target for the treatment of type 2 diabetes." Current Diabetes Reports **18**(8): 55.

Baynes, H. W. (2015). "Classification, pathophysiology, diagnosis and management of diabetes mellitus." J diabetes metab **6**(5): 1-9.

Bekris, L. M., et al. (2010). "APOE mRNA and protein expression in postmortem brain are modulated by an extended haplotype structure." American Journal of Medical Genetics Part B: Neuropsychiatric Genetics **153**(2): 409-417.

Bizzarro, A., et al. (2009). "The complex interaction between APOE promoter and AD: an Italian case–control study." European journal of human genetics **17**(7): 938-945.

Chaaba, R., et al. (2008). " Association between apolipoprotein E polymorphism, lipids, and coronary artery disease in Tunisian type 2 diabetes." Journal of clinical lipidology **2(5)**: 360-364.

Chaudhary, R., et al. (2012). "Apolipoprotein E gene polymorphism: effects on plasma lipids and risk of type 2 diabetes and coronary artery disease." Cardiovascular diabetology **11**(1): 36.

Choi, K. Y., et al. (2019). "APOE Promoter Polymorphism-219T/G is an Effect Modifier of the Influence of APOE ε4 on Alzheimer's Disease Risk in a Multiracial Sample." Journal of clinical medicine **8**(8): 1236.

Craig, M. E., et al. (2014). "Definition, epidemiology, and classification of diabetes in children and adolescents." Pediatric diabetes **15**(S20): 4-17.

Da Wei, C., et al. (2019). "Association between ApoE polymorphism and type 2 diabetes: A meta-analysis of 59 studies." Biomedical and Environmental Sciences **32**(11): 823-838.

Dan T A Eisenberg and C. W. K., M Geoffrey Hayes (2010). "Worldwide Allele Frequencies of the Human Apolipoprotein E Gene: Climate, Local Adaptations, and Evolutionary History " Am J Phys Anthropol **143(1)**: 100-111.

Das, H., et al. (2018). "Classification of Diabetes Mellitus Disease (DMD): A Data Mining (DM) Approach." Progress in Computing, Analytics and Networking: *539–549.*

Dankner, R., et al. (2020). "ApoE genotype, lipid profile, exercise, and the associations with cardiovascular morbidity and 18-year mortality." The Journals of Gerontology: Series A **75**(10): 1887-1893.

Elmadbouh, I., et al. (2013). "Relationship of apolipoprotein E polymorphism with lipid profiles in atherosclerotic coronary artery disease." The Egyptian Heart Journal **65**(2): 71-78.

Erdogan, M., et al. (2009). "The relationship of the apolipoprotein e gene polymorphism in Turkish Type 2 diabetic patients with and without nephropathy." Journal of endocrinological investigation **32**(3): 219-222.

Eto, M., et al. (2002). "Apolipoprotein E genetic polymorphism, remnant lipoproteins, and nephropathy in type 2 diabetic patients." American journal of kidney diseases **40**(2): 243-251.

Fan, W. (2017). "Epidemiology in diabetes mellitus and cardiovascular disease." Cardiovascular endocrinology **6**(1): 8.

Fritsche, L. G., et al. (2009). "Age-related macular degeneration and functional promoter and coding variants of the apolipoprotein E gene." Human mutation **30**(7): 1048-1053.

Galal, A. A., et al. (2020). "Association of Apolipoprotein E gene polymorphism with the risk of T2DM and obesity among Egyptian subjects." Gene: 145223.

Geng, H., et al. (2011). "APOE genotype-function relationship: Evidence of− 491 A/T promoter polymorphism modifying transcription control but not type 2 diabetes risk." PLoS One **6**(10): e24669.

Ghandour, R., et al. (2018). "Complications of type 2 diabetes mellitus in Ramallah and al-Bireh: the palestinian diabetes complications and control study (PDCCS)." Primary Care Diabetes **12**(6): 547-557.

Goldberg, T. E., Huey, E. D., & Devanand, D. P. (2020). "Association of APOE e2 genotype with Alzheimer's and non-Alzheimer's neurodegenerative pathologies." Nature communications **11**(1): 1-8.

Gurinovich, A., et al. (2019). "Varying effects of APOE alleles on extreme longevity in European ethnicities." The Journals of Gerontology: Series A **74**(Supplement_1): S45-S51.

Hu, Y., et al. (2010). "Combined use of fasting plasma glucose and glycated hemoglobin A1c in the screening of diabetes and impaired glucose tolerance." Acta diabetologica **47**(3): 231-236.

Huang, M., et al. (2020). "APOE rs405509 polymorphism and Parkinson's disease risk in the Chinese population." Neuroscience Letters **736**: 135256.

Jiang, Y., et al. (2017). "Effects of Apolipoprotein E isoforms in diabetic nephropathy of Chinese type 2 diabetic patients." Journal of Diabetes Research **2017:** 1-6.

Karlsson IK., et al. (2018). "Apolipoprotein E DNA methylation and late-life disease." Int J Epidemiol **47**(3):899-907

Katsarou, A., et al. (2017). "Type 1 diabetes mellitus." Nature reviews Disease primers **3**(1): 1-17.

Khabour, O. F. and E. S. Abdelhalim (2020). "Distribution of APOE gene variations in the Jordanian population: Association with longevity." Journal of King Saud University-Science **32**(1): 518-522.

Kharroubi, A. T., and Darwish, H. M (2015). "Diabetes mellitus: The epidemic of the century." World journal of diabetes **6**(6): 850.

Kim, C., et al. (2002). "Gestational diabetes and the incidence of type 2 diabetes: a systematic review." Diabetes care **25**(10): 1862-1868.

Kim, H.-Y. (2017). "Statistical notes for clinical researchers: Chi-squared test and Fisher's exact test." Restorative dentistry & endodontics **42**(2): 152-155.

Lambert, J.-C., et al. (1998). "A new polymorphism in the APOE promoter associated with risk of developing Alzheimer's disease." Human molecular genetics **7**(3): 533-540.

Laws, S. M., et al. (2003). "Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription." Journal of neurochemistry **84**(6): 1215-1236.

Li, H., et al. (2008). "Variants in the fat mass–and obesity-associated (FTO) gene are not associated with obesity in a Chinese Han population." Diabetes **57**(1): 264-268.

Little, R. R., et al. (2019). "The National Glycohemoglobin Standardization Program: over 20 years of improving hemoglobin A1c measurement." Clinical chemistry **65**(7): 839-848.

Livny, A., et al. (2016). "Long-term variability in glycemic control is associated with white matter hyperintensities in APOE4 genotype carriers with type 2 diabetes." Diabetes care **39**(6): 1056-1059.

Lopez-Lopez, J., et al. (2018). "The simultaneous assessment of glycosylated hemoglobin, fasting plasma glucose and oral glucose tolerance test does not improve the detection of type 2 diabetes mellitus in Colombian adults." PLoS One **13**(4): e0194446.

Lou, L., et al. (2019). Genetic associations between Transcription Factor 7 Like 2 rs7903146 polymorphism and type 2 diabetes mellitus: a meta-analysis of 115,809 subjects. Diabetology & Metabolic Syndrome **11**(1): 56.

Mannila, M. N., et al. (2013). "Identification of a functional apolipoprotein E promoter polymorphism regulating plasma apolipoprotein E concentration." Arteriosclerosis, thrombosis, and vascular biology **33**(5): 1063-1069.

Margeta, M. A., et al. (2020). "Association of APOE With Primary Open-Angle Glaucoma Suggests a Protective Effect for APOE ε4." Investigative ophthalmology & visual science **61**(8): 3-3.

Maureen I. Harris, et al. (1995)." Diabetes in America." National Institute of Health NIH Pub No. 17-1468. 3rd Ed.

Mauricio, D., et al. (2020). "Chronic diabetes complications: the need to move beyond classical concepts." Trends in Endocrinology & Metabolism **31**(4): 287-295.

McIntyre, H. D., et al. (2019). "Gestational diabetes mellitus." Nature reviews Disease primers **5**(1): 1-19.

Oguntibeju, O. O. (2019). "Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links." International journal of physiology, pathophysiology and pharmacology **11**(3): 45.

Ogurtsova, K., et al. (2017). "IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040." Diabetes research and clinical practice **128**: 40-50.

Palombo, C. and Kozakova, M. (2020). "Lipids and Cardiovascular Organ Damage in Type 2 Diabetes Mellitus." Cardiology and Cardiovascular Medicine **4**(4): 346-360.

Ravona-Springer, R., et al. (2014). "The ApoE4 genotype modifies the relationship of long-term glycemic control with cognitive functioning in elderly with type 2 diabetes." European Neuropsychopharmacology **24**(8): 1303-1308.

Roglic, G. (2016). "WHO Global report on diabetes: A summary." International Journal of Noncommunicable Diseases **1**(1): 3.

Sabarneh, A., et al. (2018). "Common FTO rs9939609 variant and risk of type 2 diabetes in Palestine." BMC Medical Genetics **19**(1): 156.

Sapkota, B., et al. (2015). "Association of APOE polymorphisms with diabetes and cardiometabolic risk factors and the role of APOE genotypes in response to anti-diabetic therapy: results from the AIDHS/SDS on a South Asian population." Journal of diabetes and its complications **29**(8): 1191-1197.

Selvin, E., et al. (2010). "Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults." New England Journal of Medicine **362**(9): 800-811.

Shah, S. M., et al. (2017). "Prevalence of diabetes among migrant women and duration of residence in the United Arab Emirates: a cross sectional study." PLoS One **12**(1): e0169949.

Shi, J., et al. (2020). "ε2 allele and ε2-involved genotypes (ε2/ε2, ε2/ε3, and ε2/ε4) may confer the association of APOE genetic polymorphism with risks of nephropathy in type 2 diabetes: a meta-analysis." Lipids in Health and Disease **19**(1): 1-14.

Singh, P., et al. (2006). "APOE distribution in world populations with new data from India and the UK." Annals of human biology **33**(3): 279-308.

Sircana, A., et al. (2018). "Altered gut microbiota in type 2 diabetes: just a coincidence?" Current Diabetes Reports **18**(10): 98.

Szumilas, M. (2010). "Explaining odds ratios." Journal of the Canadian academy of child and adolescent psychiatry **19**(3): 227.

Tashrifi, F., et al. (2018). "Fasting Blood Sugar, Glucose Challenge Test and One-Two Hour Glucose Tolerance Test in Diagnosis of Gestational Diabetes in Women without Risk Factor." Iranian Journal of Diabetes and Obesity **10**(2): 106-108.

van den Bosch, M., et al. (2019). "More severe OA pathology in a humanized mouse model for APOE-ε4 as compared to APOE-ε3: APOE-isoforms as possible risk factor for inflammatory osteoarthritis development?" Osteoarthritis and Cartilage **27**: S372.

Weber, K. S., et al. (2016). "Associations between explorative dietary patterns and serum lipid levels and their interactions with ApoA5 and ApoE haplotype in patients with recently diagnosed type 2 diabetes." Cardiovascular diabetology **15**(1): 138.

Wong, M. W. K. B., et al. (2019). "APOE genotype differentially modulates plasma lipids in healthy older individuals, with relevance to brain health." Journal of Alzheimer's Disease **72(3)**: 703-716.

Wu, Y., et al. (2014). "Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention." International journal of medical sciences **11**(11): 1185.

Yin, Y.-W., et al. (2014). "Influence of apolipoprotein E gene polymorphism on development of type 2 diabetes mellitus in Chinese Han population: a meta-analysis of 29 studies." Metabolism **63**(4): 532-541.

Zhang, C., et al. (2015). "Association of ApoE gene with type 2 diabetic nephropathy in a Chinese population: a meta-analysis of case-control studies." Annales d'endocrinologie, Elsevier **76**(5): 601-613.

Zhang, P., et al. (2017). "Apolipoprotein status in type 2 diabetes mellitus and its complications." Molecular medicine reports **16**(6): 9279-9286.

Zhen, J., et al. (2018). "Association of ApoE genetic polymorphism and type 2 diabetes with cognition in non-demented aging Chinese adults: a community based cross-sectional study." Aging and disease **9**(3): 346.

Zhong, L., et al. (2016). "A rapid and cost-effective method for genotyping apolipoprotein E gene polymorphism." Molecular neurodegeneration **11**(1): 1-8.

Zhou, T., et al. (2020). "Association of apoE gene polymorphisms with lipid metabolism in renal diseases." African Health Sciences **20(3)**: 1368-1381.

Appendices

Appendix 1: Research approval by research ethics committee at Al-Quds University.

Al-Quds University
Jerusalem
Deanship of Scientific Research

بسم الله الرحمن الرحيم

جامعة القدس
القدس
عمادة البحث العلمي

Research Ethics Committee
Committee's Decision Letter

Date: April 20, 2019
Ref No: 71/REC/2019

Dear Dr Suheir Ereqat,

Thank you for submitting your application for research ethics approval. After reviewing your application entitled **"Genetics and Epigenetics of Type 2 Diabetes Mellitus: The role of APOE gene."** The Research Ethics Committee confirms that it is in accordance with the research ethics guidelines at Al-Quds University. Please inform us if there will be any changes in your research methodology, subjects, plan and we would appreciate receiving a copy of your final research report.
Thank you again and wish you productive research that serves the best interest of your subjects.

Dina M. Bitar PhD
Research Ethics Committee Chair

Cc. Prof. Imad Abu Kishek - President
Cc. Members of the committee
Cc. file

Appendix2: DNA extraction protocol.

The procedure was done as followed:

1. 25 µL Proteinase K were added to 200 µL blood in a 1.5 mL microcentrifuge tube.

2. 200 µl Buffer B3 were added to each sample, mixed using vortex for (10-20)s.

3. Samples' mixtures were incubated at 70 °C for 10–15 min.

4. 210 µl Ethanol (96%-100%) were added to each sample and immediately vortexed for 10-15 seconds.

5. The resulted mixture was loaded carefully to the NucleoSpin® Tissue Column placed in a collection tube. Centrifugation was done at 11,000 x g for 1 min. Collection tube was discarded with flow-through and the column was placed in a new Collection Tube.

6. 500 µl BW Buffer were added to the NucleoSpin® Tissue Column and centrifuged at 11,000 xg for 2 min. Collection tube was replaced with a new one.

7. 600 µl Buffer B5 were added and the tubes were centrifuged at 11,000 x g for 3 min. Thendiscard flow-through and reuse Collection Tube.

8. The NucleoSpin® Tissue Column was placed in a clean microcentrifuge tube, addition of 100µl of preheated buffer BE (70◦C) to the column and centrifugation at 11000 x g for 1 min was done.

10. Buffer was directly dispensed onto the silica membrane. Samples were incubated at room temperature for 1 min and then centrifuged for 1 min at 11,000 x g. The obtained DNA was stored at -20◦C until further analysis.

Appendix3: Sequences of APOE gene and promoter revealing the location of primers and SNPs.

APOE allele analysis (Gene)

>NM_001302688.1 Homo sapiens apolipoprotein E (APOE), transcript variant 1, mRNA

TCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCGCCTGGT
GCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTC
CCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTAC
CAGGCCGGGGC

Shaded with yellow: primers (two forward and the last is the reverse).

Shaded with blue: single nucleotide polymorphisms

APOE promoter analysis

gi|568815579|ref|NC_000019.10|:44904000-44906670 Homo sapiens chromosome 19, GRCh38.p12 Primary Assembly
CCTCCCATCCCACTTCTGTCCAGCCGCCTAGCCCCACTTTCTTTTTTTTCTTTTTTTGAGACAGTCTCC
CTCTTGCTGAGGCTGGAGTGCAGTGGCGAGATCTCGGCTCACTGTAACCTCCGCCTCCCGGGTTCAAGC
GATTCTCCTGCCTCAGCCTCCCAAGTAGCTAGGATTACAGGCGCCCGCCACCACGCCTGGCTAACTTTT
GTATTTTTAGTAGAGATGGGGTTTCACCATGTTGGCCAGGCTGGTCTCAAACTCCTGACCTTAAGTGAT
TCGCCCACTGTGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCTACCGCCCCCAGCCCCTCCCATCC
CACTTCTGTCCAGCCCCCTAGCCCTACTTTCTTTCTGGGATCCAGGAGTCCAGATCCCCAGCCCCCTCT
CCAGATTACATTCATCCAGGCACAGGAAAGGACAGGGTCAGGAAAGGAGGACTCTGGGCGGCAGCCTCC
ACATTCCCCTTCCACGCTTGGCCCCCAGAATGGAGGAGGGTGTCTGTATTACTGGGCGAGGTGTCCTCC
CTTCCTGGGGACTGTGGGGGGTGGTCAAAAGACCTCTATGCCCCACCTCCTTCCTCCCTCTGCCCTGCT
GTGCCTGGGGCAGGGGGAGAACAGCCCACCTCGTGACTGGGGGCTGGCCCAGCCCGCCCTATCCCTGGG

Shaded with red: single nucleotide polymorphisms.

Shaded with Gray: 1st forward primer/yellow: 2nd forward /purple: 3rd forward (used in the repeated conventional PCR after the weak results came from sequencing of the multiplex PCR)/green: reverse.

Appendix4: Sequence of forward primer used in the repeated conventional PCR for the promoter SNP rs405509 A/C.

PCR-Pr	sequence
SNPpromoterAPOER	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTGGATCCCAGAAAGAAA3'

The shaded sequence is the adapter.

Appendix5: Gel preparation steps.

1. 2 grams of agarose (2%) were weighed and placed in a glass beaker. Then 100 ml of TAE buffer were added.

2. The mixture was boiled until it became well dissolved and clear. Agarose suspension was allowed to be cooled to about 40°C before pouring it.

3. Two drops of Ethidium bromide were added to the solution, mixed carefully and poured into agarose gel casting system (Bio-Rad, SUB-CELL®GT). A desired comb was inserted.

4. The comb was gently removed.

5. The gel was placed in an electrophoresis chamber and covered with TAE buffer until wells are submerged.

6. 5 µl of DNA were loaded into the wells.

7. We put the cover in which the cathode was to the well side and the anode to the other one.

8. The voltage used was 120 volts for 30 min using Bio-Rad power supply, POWER PAC 3000.

9. Agarose gel was placed over the UV transilluminator device to be exposed to UV light to see the bands and photos were taken.

المتغيرات الجينية لـ APOE وخطر إرتفاع نسبة الدهون لدى مرضى السكري من النوع 2 في المجتمع الفلسطيني

إعداد: منال محمد صالح غطاس

إشراف: د.سهير عريقات

المشرف الثاني: د. عبدالمجيد نصرالدين

الملخص

مرض السكري هو من أكثر الأمراض الأيضية انتشاراً في العالم. تسعون بالمئة من مصابي السكري هم من النوع الثاني (T2DM). الخلل الذي يؤدي إلى تطور المرض هو مقاومة الانسولين أو عدم افرازه مما يؤدي إلى زيادة نسبة الجلوكوز في الدم عن المعدل الطبيعي. هناك العديد من العوامل التي قد تكون سبباً في الإصابة بالمرض وهي قد تكون بيئية أو جينية أو الاثنتان معاً. هناك عدة جينات تلعب دوراً في زيادة خطر الإصابة بمرض السكري لدى حامليها. واحداً من هذه الجينات هو (*APOE*) جين وهو المسؤول عن إنتاج الأبوليبوبروتين E والذي له أهمية في عملية التمثيل الغذائي للدهون.

هذه الدراسة المستعرضة تمت في الفترة بين شهر كانون الثاني إلى نيسان من عام 2019. اشترك في الدراسة 204 مرضى، قُسِّموا إلى مجموعتين: الأشخاص الذين يعانون من الدهون وعددهم 96 والأشخاص غير المصابين بالدهون وعددهم 108. كل المرضى المشاركين أعمارهم تتجاوز الخمسين عاماً وكانوا قد أُدخلوا إلى مستشفى رام الله الحكومي في فلسطين. تم قياس مستوى الدهون خلال الفحوصات الطبية في المستشفى. اثنان من النيوكليوتيدات المفردة المختلفة في داخل الإكسون الرابع من APOE جين وثلاثة نيوكليوتيدات مفردة مختلفة في منطقة المحفز ل APOE جين تم

تحديدها باستخدام تسلسل الجين القادم وهذه الدراسة هي الأولى من نوعها في فلسطين. التكرارات لهذه النيوكليوتيدات المنفردة تم إيجادها للمجتمع المدروس. بالإضافة إلى أنه تمت دراسة العلاقة بين كل من الطرز الجينية ل APOE جين والنيوكليوتيدات المفردة لمحفز الجين وخطر الإصابة بإرتفاع الدهنيات عند مرضى السكري من النوع الثاني. نتائج دراستنا أظهرت أن التكرار للأليل E3 (79.6%) كان الأعلى بين الأليلات الأخرى، والطراز الجيني E3/E3 كان أيضاً له التكرار الأعلى بين كل مرضى السكري المشاركين بنسبة 78.1%. تم تقسيم المجتمع المدروس حسب نسبة الدهون، النتائج أظهرت أنه لا يوجد اختلاف في التكرارات لكل من الطرز الجينية ل APOE والنيوكليوتيدات المفردة للمحفز بين المجموعتين: المصابين بالدهنيات وغير المصابين. وأيضاً تحليل الانحدار اللوجستي المعدل حسب العمر والجنس ومؤشر كتلة الجسم أظهر عدم وجود علاقة بين كل من جين ال APOE والنيوكليوتيدت المفردة لمحفزه وخطر الإصابة باتفاع الدهون. كما أظهرت النتائج أن لا اختلافاً بين الطرز الجينية ل APOE ومعدل كل من الكوليستيرول، الدهون الثلاثية، البروتين الدهني مرتفع الكثافة والبروتين الدهني منخفض الكثافة. كما أنه تم الكشف عما إذا كان هناك علاقة بين الطرز الجينية ل APOE والنيوكليوتيدات المنفردة لمحفز الجين بين كل من المجموعتين المقسمتين ولكن أيضاً لم يوجد علاقة.

في الختام، العلاقة بين الجينات والأمراض تختلف حسب العرق. نتائج بحثنا أظهرت أنه لا يوجد علاقة بين جين APOE ونسبة الدهون لدى مرضى السكري في المجتمع الفلسطيني. لتأكيد هذه الدراسة، يجب عمل دراسات أخرى شبيهة ولكن على عينة ذات حجم أكبر.