The Islamic University – Gaza
Deanery of Higher Education
Faculty of Science
Master of Biological Sciences
Medical Technology

الجامعة الإسلامية – غزة
عمادة الدراسات العليا
كلية العلوم
ماجستير العلوم الحياتية
تحاليل طبية

Calpain 10 Gene Polymorphism in Type 2 Diabetes Mellitus Patients in Gaza Strip

Prepared by

Mazen M. El Zaharna

Supervisors:

Prof. Fadel A. Sharif **Dr. Abdalla Abed**

Submitted in Partial Fulfillment of Requirements for the Degree of
Master of Biological Sciences/Medical Technology
Department of Medical Technology
Faculty of Science

2007 م / 1428 هـ

بسم الله الرحمن الرحيم

الجامعة الإسلامية – غزة
The Islamic University - Gaza

عمادة الدراسات العليا **هاتف داخلي: 1150**

الرقم Ref. ج س غ/35/.....
التاريخ Date 2007/05/05.....

نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة عمادة الدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحث/ **مازن مدحت مصطفى الزهارنة** لنيل درجة الماجستير في **كلية *العلوم*/ قسم العلوم الحياتية–تحاليل طبية** وموضوعها:

"Calpain 10 Gene Polymorphism in Type 2 Diabetes Mellitus Patients in Gaza Strip"

وبعد المناقشة العلنية التي تمت اليوم الثلاثاء 21 ربيع آخر 1428هـ، الموافق 2007/05/08م الساعة الواحدة ظهراً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

أ.د. فضل الشريف	مشرفاً ورئيساً	.....
د. عبد الله عابد	مشرفاً	.....
أ.د. ماجد ياسين	مناقشاً داخلياً	.....
د. محمود سرداح	مناقشاً خارجياً	.....

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

واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق ،،،

عميد الدراسات العليا

د. مازن إسماعيل هنية

ص.ب 108 الرمال، غزة فلسطين تلفون tel: +970 (8) 286 0700 فاكس fax: +970 (8) 286 0800 P.O. Box 108, Rimal, Gaza, Palestine
www.iugaza.edu.ps public@iugaza.edu.ps

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university of other institute, except where due acknowledgment has been made in the text.

Signature	Name	Date
Mazen	Mazen Medhat Moustafa El Zaharna	March- 28 - 2007

Dedication

To my father and my mother who taught me how to give

To my wife who supported me wholeheartedly

To my children Medhat & Motasem

To my brothers and sisters

To all my friends who spare no effort to help

To all of them I dedicate this work

Acknowledgements

The work for this thesis was carried out at the Molecular biology Laboratory-Islamic University – Gaza.

Many individuals have contributed to the completion of this work, including the formation of this dissertation. I wish to thank all who made this work possible with their support, advice, and time.

I would like to express my gratitude to my supervisors Prof. Fadel A. Sharif and Dr. Abdalla Abed, for their support, patience, special insights and encouragement throughout my research. The technical and editorial advice of Prof. Fadel A. Sharif was essential to the completion of this work and have taught me innumerable lessons and insights on the workings of academic research in general.

I am very much thankful to the staff at the Diabetic Clinic – Al Remal Clinic for their help in sample collection. Especially Dr. Soheal El Kichoui, Dr. Samy El Esawi, Dr. Mohammed Bolbol, Dr. Awny El Jaro and Mr. Yosef Abu Rahma. Their sincere contributions made this research attempt fruitful and possible**.**

I am deeply appreciate the assistance and support of Mr. Nasser Abu Shaaban, in the technical work.

I want to thank all the staff at the department of the biological sciences at the Islamic University- Gaza, for their support and help. I would like also to warmly thank Dr. Abboud El Kichaoui for his help and support during the course. I would like also to thank Mr. Mohamed Abou Oada for his assistance.

I want to thank all my colleagues, especially Zaid, Iyad, and Hosam thank you so much for always being there for me offering a helping hand. Good luck with everything!

Last but not least, I want to thank my beloved father and mother, thank you for always believing in me and loving me unconditionally, without your support and love I would not be where I am today. My heartfelt thanks go to my loving wife; your tender care and patience have not ceased even in my worst moments

To all of these individuals I owe many thanks for their insights and unlimited support

Abstract

Calpain-10 (*CAPN10*) is a member of a large family of intracellular proteases. The polymorphisms at *CAPN10* gene have recently been associated with complex forms of type 2 diabetes mellitus (T2DM). It was shown in Mexican-Americans and other populations that variants of polymorphisms, single nucleotide polymorphism (SNP)-44, SNP-43, deletion/insertion (del/ins)-19, and SNP-63, of this gene influence susceptibility to T2DM. However, substantial differences were shown between ethnic groups in at risk alleles and haplotypes as well as in their attributable risk.

Aim: To examine the role of *CAPN10* SNPs -44, -43, -63, and del/ins-19 in genetic susceptibility to T2DM and to the levels of triglycerides and cholesterol in Gaza Strip.

Methods: Ninety six individuals were examined: 48 T2DM patients and 48 control individuals. The groups were genotyped for *CAPN10* SNP-44, SNP-43, SNP-63, and del/ins-19. Mutagenically separated polymerase chain reaction (MS-PCR) was used to examine SNP-44, del/ins-19 was examined by electrophoresis of the PCR product on agarose gel by size, while the restriction fragment length polymorphism (RFLP) method was used for SNP-43 and SNP-63.

Results: There was no association between T2DM and allele frequencies of SNP-43, del/ins-19 and SNP-63. Distributions of genotypes, haplotypes and haplotypes combinations in the studied variants were not significantly different between the groups. However, there was evidence that the C-allele at SNP-44, plays a possible role in the susceptibility to T2DM (p=0.01). T2DM patients with G/A genotype were found to have higher cholesterol levels in comparison to those homozygous for allele 1 (G/G) in SNP-43. We found also that T2DM patients who are homozygous for del/ins-19 allele 2 have higher cholesterol levels in comparison to other genotypes. In addition, T2DM patients having the 121/221 haplotypes combination have higher serum cholesterol level than those with other haplotypes combinations. And that control subjects with haplotypes

combination 111/121 have higher serum cholesterol level than those with other haplotypes combinations.

Conclusion: The polymorphism SNP-44 at *CAPN10* has an association with T2DM. *CAPN10* also has an association with the levels of cholesterol in both T2DM patients and control individuals.

Keywords: Calpain 10, T2DM, Triglycerides, Cholesterol, Gaza strip.

التعدد الشكلي في الجين كالبين-10 لدى مرضى السكري من النوع الثاني في قطاع غزة

الملخص

ارتبط التعدد الشكلي في الجين كالبين-10 (Calpain-10) في الآونة الأخيرة بمرض السكري من النوع الثاني. يعتبر البروتين الناتج عن هذا الجين عضوا من أسرة كبيرة من الإنزيمات الهاضمة للبروتينات الموجودة داخل الخلية. هذا وقد تبين من الدراسات علي الأمريكيين من أصل مكسيكي وغيرهم من السكان في دول أخرى أن التعدد الشكلي للجين كالبين-10، المتمثل في: [الطفرة أحادية النكليوتيدة (SNP)-44، SNP-43، الطفرة حذف/إضافة (del/ins)-19، و SNP-63]، يؤثر على قابلية الإصابة بمرض السكري من النوع الثاني. و مع ذلك، وجدت اختلافات كبيرة بين المجموعات العرقية في الألائل (Alleles) و الأنماط الفردانية (Haplotypes)، التي تشكل الخطر المعزو.

الهدف : دراسة دور التغيرات في الجين كالبين-10؛ و المتمثلة بالطفرات: SNP-44، SNP-43، del/ins-19، و SNP-63 ؛ و الاستعداد الوراثي للإصابة بمرض السكري من النوع الثاني و أيضا بارتفاع مستويات الدهون الثلاثية و الكولسترول في قطاع غزة.

الطرق: تم فحص عينة مكونة من 96 شخص و مقسمة إلي مجموعتين، المجموعة الأولي مكونة من 48 شخص مصابين بمرض السكري من النوع الثاني، و المجموعة الثانية مكونة من 48 شخص طبيعي (غير مصاب بالسكري) كعينة ضابطة. و قد تم دراسة النمط الجيني (Genotype) في المجموعتين للتغيرات SNP-44، SNP-43، SNP-63 و del/ins-19 في الجين كالبين-10. هذا و قد تم استخدام تقنية MS-PCR لفحص SNP-44، و تقنية الهجرة الكهربائية للناتج PCR علي جل الأجاروز بواسطة الحجم ل del/ins-19، بينما تم استخدام تقنية RFLP-PCR لكل من SNP-43 و SNP-63.

النتائج : أظهرت نتائج هذه الدراسة عدم وجود علاقة معنوية بين مرض السكري من النوع الثاني و الترددات في الألائل لكل من SNP-43، del/ins-19، و SNP-63. هذا و أشارت نتائج هذه الدراسة أيضا إلي عدم وجود فروقات ذات دلالة إحصائية في النمط الجيني، الأنماط الفردانية و تجميع الأنماط الفردانية (Haplotypes combinations) بين المجموعتين، في التغيرات التي تم دراستها. كما بينت النتائج أن وجود أليل (C) في SNP-44 يمكن أن يكون له دور في احتمالية التعرض للإصابة بمرض السكري من النوع الثاني. هذا و بينت النتائج أن مرضى السكري من النوع الثاني الذين يحملون النمط الجيني (G/A) في SNP-43 أن مستوى الكولسترول لديهم أعلى بالمقارنة مع أولئك الذين يحملون النمط الجيني (G/G). هذا و بينت النتائج أيضا أن مرضى السكري من النوع الثاني الذين يحملون ألائل متماثلة للأليل رقم 2 في del/ins-19 أن مستويات الكولسترول لديهم أعلى من أولئك

الذين يحملون الألائل الأخرى. و قد أظهرت النتائج أيضا أن مرضى السكري من النوع الثاني الذين يحملون haplotypes combination (121/221) لديهم مستويات الكولسترول أعلى من أولئك الذين يحملون Haplotypes Combinations الأخرى. و أن الأشخاص الطبيعيين الذين يحملون (111/121) Haplotypes combinations لديهم مستويات الكولسترول أعلي من أولئك الذين يحملون Haplotypes Combination الأخرى.

الخلاصة: لقد بينت نتائج هذه الدراسة وجود علاقة بين SNP-44 في الجين كالبين-10 و مرض السكري من النوع الثاني، كما بينت أيضا وجود علاقة بين هذا الجين و ارتفاع مستويات الكولسترول في بلازما الدم.

Table of Contents

Contents **Page**

C h a p t e r 3 – Materials and Methods

Chapter 4 – Results

Chapter 5 – Discussion

List of Figures

List of Tables

Abbreviations

ATP	Adenosine triphosphate
BMI	Body mass index
CAPN10	Calpain 10
CVD	Cardiovascular disease
Del/ins	Deletion/insertion
DM	Diabetes mellitus
DZ	Dizygotic
FABP2	Fatty-acid binding protein 2
FFA	Free fatty acids
GDM	Gestational diabetes mellitus
HGP	Hepatic glucose production
HLA	Human leukocyte antigen
HNF4A	Hepatocyte nuclear factor 4, alpha
HW	Hardy-Weinberg
IDDM	Insulin-dependent diabetes mellitus
IFG	Impaired fasting glucose
IGF-1	Insulin-growth factor 1
IGT	Impaired glucose tolerance
K-ATP	Sensitive potassium channel adenosine triphosphate
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
kD	Kilo dalton
LD	Linkage disequilibrium
MODY	Maturity-onset diabetes in the young
MZ	Monozygotic
NGT	Normal glucose tolerance
NIDDM1	Non-insulin-dependent diabetes mellitus-1
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
PPARG	Peroxisome proliferative activated receptor, gamma
RFLP	Restriction fragment length polymorphism
rpm	Round per minute
RT	Room temperature
RT–PCR	Reverse transcription polymerase chain reaction
SNP	Single nucleotide polymorphism
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UCP2	Uncoupling protein 2
WBC	White blood cells
WHO	The world health organization
UV	Ultraviolet

Chapter 1 Introduction

Diabetes Mellitus (DM) is a progressive and chronic endocrine disorder which results primarily in a hyperglycemic (excess glucose in the blood) condition (1). DM affects the body's ability to metabolize fat, carbohydrates and proteins and often leads to a serious micro- and macrovascular complications, including cardiovascular diseases (2). The primary hormone, insulin, which maintains homeostasis of body glucose levels, is either insufficient or ineffective in individuals with DM (3).

1.1 Classification of diabetes mellitus

The most common types of diabetes mellitus are:

1. Type 1 diabetes dellitus (T1DM) results from autoimmunological destruction of the insulin-producing cells of the pancreas and accounts for 5–10% of all cases of diabetes, with the major susceptibility gene mapping to the human leukocyte antigen (HLA) region of chromosome 6 (4).
2. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for approximately 90% of cases and affecting 10–20% of those over 45 years of age in many developed countries (4). T2DM indicates an individual who has a physiological resistance to the effects of insulin within the peripheral tissues. Basically, the insulin, which the body is still capable of producing, is not physiologically effective (5).

1.2 Etiology of type 2 diabetes mellitus

T2DM is a classic example of a complex disorder. It is strongly familial, but clearly arises as a consequence of the actions and interactions of many genetic and non-genetic factors with some forms of the disease resulting from mutations in a single gene, others are multifactorial in origin (4,6).

1.2.1 Risk factors of T2DM

The risk factors for T2DM include environmental influences such as obesity (especially abdominal obesity), aging, ethnicity, family history of diabetes, history of gestational diabetes, sedentary lifestyle, low birth weight, and polycystic ovary syndrome (7). Eighty percent or more of the people with T2DM are obese with the remaining twenty percent considered above ideal weight indicating obesity as a predominant link to the development of T2DM (8).

1.2.2 Type 2 diabetes mellitus and genetics

Although insulin resistance and progressive pancreatic β-cell dysfunction have been established as the two fundamental features in the pathogenesis of T2DM, the specific molecular defects affecting insulin sensitivity and/or β-cell function remain largely undefined (2).

Substantial scientific evidence exists for the role of genetic factors in the pathogenesis of T2DM. For example, T2DM clusters in families, its concordance rate in monozygotic twins is higher than in dizygotic ones, and there are ethnic groups with a very high prevalence of this disease (9).

Although genomewide scans have identified several potential chromosomal susceptibility regions in several human populations, finding a causative gene for T2DM has remained elusive. Hanis et al. (1996) reported linkage to a region on chromosome 2q37.3 among Mexican Americans and identified a major susceptibility locus, located in the interval that spans markers D2S125–D2S140 (10). Following a combined strategy of positional cloning and a newly developed statistical method of partitioning linkage, these investigators identified a novel gene, Calpain 10 (*CAPN10*, as a putative T2DM susceptibility gene in this region (Horikawa et al. 2000) (4). Polymorphisms in this gene (SNP-44, SNP-43, del/ins-19, and SNP-63), all located in intronic sequences, were found to be involved in increased risk of the disease. Although the common G allele at SNP-

43 was initially found to be significantly associated with the phenotype in families that showed linkage to NIDDM1 region on chromosome 2 (11). Variation in *CAPN10* has been associated with a threefold increased risk of T2DM in Mexican-Americans and an increased risk of diabetes in Northern European populations (12).

The understanding of the genetic basis of T2DM would assist in the development of screening tests to identify subjects at risk of developing T2DM at an early stage so that prevention strategies, lifestyle advice, and medical treatment can be commenced at the earliest possible stage. In addition, an understanding of the genetic basis of T2DM will pave the way for discovering new approaches for the prevention and more effective treatment of this condition (13).

1.3 Aim of the study

The overall aim of the present study is to assess calpain-10 gene polymorphisms in type 2 diabetes mellitus patients in Gaza strip.

The specific Objectives are:

1. To study the prevalence of common variants in *CAPN10* gene, SNPs –44, -43, -63 and del/ins-19 in T2DM as compared to control subjects
2. To evaluate the relation of these variants in the development of T2DM in Gaza Strip population
3. To investigate the influence of these variants on the lipid profile in T2DM patients and control group

Chapter 2 Literature Review

2.1 Prevalence of diabetes mellitus

Changes in human behaviour and lifestyle over the last century have resulted in a dramatic increase in the incidence of diabetes worldwide. The number of adults with diabetes in the world is expected to rise from 135 million in 1995 to 300 million in the year 2025 (14). Current estimates from different countries in Europe and the United States have shown that diabetes and its complications account for 8 to 16% of the total health costs for society and these will increase dramatically unless major efforts are made to prevent the ongoing epidemic (14). Worldwide prevalence of diabetes in the year 2003 and the predicted prevalence in the year 2025 are shown in Table 2.1 (15).

Table 2.1. Worldwide prevalence of diabetes in year 2003 and 2025 predicted.

	Prevalence (n x 10^6)		Increase %
	2003	**2025 Predicted**	
Asia	81.8	156.1	91
Europe	38.2	44.2	16
North America	25	39.7	59
South America	10.4	19.7	88
Middle East	18.2	35.9	97
Africa	13.6	26.9	98
Australia	1.1	1.7	59
Total Cases Worldwide	189	324	72

Adopted from reference number 15.

2.1.1 Prevalence of diabetes mellitus in Palestine

In Palestine, DM seems to be a serious health problem among population especially the refugees. The prevalence rate of DM in Palestine was about 9% in 2002, while the international rate was about 5.2% in the age 20-79. About

65.5% of all Palestinian diabetics had at least one of the major complications and only 39.7% had acceptable glycemic control. The mortality rate of DM in Palestine has been increased from 7.9 per 100,000 in 1995 to 15.3 per 100,000 in 2002 and decreased to 8.5 per 100,000 in 2005. The age group of ≥ 60 years old constitutes about 8.2% from the total deaths in Palestine due to DM. However, in Palestine, there is under-diagnosis and under-reporting in DM as a leading cause of death. This is due to lack of proper hospital and clinic recording systems (16).

2.2 Types of diabetes mellitus

DM is the name given to a heterogeneous group of disorders that have abnormal glucose tolerance in common. The classification given in Table 2.2 is based on etiologic differences between these disorders (17).

Table 2.2. Types of diabetes mellitus

Type	Other names used
Insulin-dependent DM (IDDM)	Type 1, juvenile-onset
Non-insulin-dependent DM (NIDDM)	Type 2, adult-onset, maturity-onset
Maturity-onset diabetes in the young (MODY)	Mason-type diabetes
Abnormal insulin structure	Insulinopathies
Insulin resistance associated with acanthosis nigricans	
Diabetes associated with congenital disorders	
Secondary diabetes	
Gestational diabetes	

Adopted from reference number 17.

The term DM describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.

The manifestation of DM include long-term damage, dysfunction and failure of various organs. DM may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long-term effects of DM include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (18).

2.2.1 Type 1 diabetes mellitus

T1DM indicates the processes of β–cell destruction that may ultimately lead to lack of insulin. Insulin is required for survival, and to prevent the development of ketoacidosis, coma and death. T1DM accounts for 5% to 10% of diabetes, and usually occurs in children or young adults (19). An individual with a T1DM process may be metabolically normal before the disease is clinically manifested, but the process of β-cell destruction can be detected. T1DM is usually characterized by the presence of anti-glutamic acid decarboxylase, islet cell or insulin antibodies which identify the autoimmune processes that lead to β–cell destruction. In some subjects with this clinical form of diabetes, particularly non–Caucasians, no evidence of an autoimmune disorder is demonstrable and these are classified as “Type 1 idiopathic” (18).

2.2.2 Type 2 diabetes mellitus

T2DM is a serious metabolic disease that affects 150 million people worldwide. It is characterized by elevation of the blood glucose concentration (20). T2DM accounts for 90% to 95% of all patients with diabetes and is increasing in

prevalence, especially in minority populations (19). Until recent years, T2DM was rarely observed in individuals under the age of 50, but increasing numbers of children are now being diagnosed with the disease. This probably reflects the growing prevalence of childhood obesity, as T2DM is exacerbated by obesity and a sedentary lifestyle (20).

People with this type of diabetes frequently are resistant to the action of insulin. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive. Ketoacidosis is infrequent in this type of diabetes; when seen it usually arises in association with the stress of another illness such as infection. Whereas patients with this form of diabetes may have insulin levels that appear normal or elevated, the high blood glucose levels in these diabetic patients would be expected to result in even higher insulin values if their β–cell function is normal. Thus, insulin secretion is defective and insufficient to compensate for the insulin resistance. On the other hand, some individuals have essentially normal insulin action, but markedly impaired insulin secretion (18).

The risk of developing T2DM increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior Gestational DM (GDM) and in individuals with hypertension or dyslipidaemia. Its frequency varies in different racial/ethnic subgroups. It is often associated with strong familial, likely genetic, predisposition. However, the genetics of this form of diabetes are complex and not clearly defined (21).

2.3 Diagnosis of diabetes

The development of T2DM is characterized by progression from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) to diabetes. Currently, T2DM is diagnosed when the underlying metabolic abnormalities consisting of insulin resistance and decreased β-cell function cause elevation of plasma glucose above 126 mg/dL (7 mmol/L) in the fasting state and/or above 200

mg/dl (11.1 mmol/L) 2 hours after 75-g oral glucose load. Persons with impaired fasting glucose (IFG) have fasting plasma glucose between 110 and 126 mg/dL (6.1 - 7 mmol/L). IGT is defined as a 2-h postprandial glucose between 140 and 200 mg/dL (7.8 - 11.1 mmol/L). IGT is more prevalent, progresses to T2DM at an annual rate of about 6-7%, and is associated with a two-fold increase in the risk for macrovascular complications (7). The criteria for the varying degrees of impairment in glucose tolerance are given in Table 2.3. These criteria are used for the diagnosis of DM but not for the type of diabetes (17).

Table 2.3. Categories of glucose tolerance impairment in non-pregnant adults

Category	**Fasting (mg/dl)**	**$^1/_2$ h, 1 h, 1$^1/_2$ h OGTT value (mg/dl)**	**2 h OGTT value (mg/dl)**
Normal	< 115[a]	< 200	< 140
IGT	< 140	≥ 200	140-200
DM	> 140[b]	≥ 200	≥ 200

Adopted from reference number 17.

[a] All values assume venous plasma glucose measured.

[b] Diagnosis of DM can be made if FBG > 140 on 2 separate days.

Many patients with T2DM are asymptomatic, and their disease is undiagnosed for many years because the hyperglycemia is often not severe enough to provoke noticeable symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications. Studies suggest that the typical patient with new-onset T2DM has had diabetes for at least 4-7 years before it is diagnosed. Among patients with T2DM, 25% are believed to have retinopathy; 9%, neuropathy; and 8%, nephropathy at the time of diagnosis (21).

2.4 Diabetes-related mortality

The death rate of men with diabetes is 1.9 times the rate for men without diabetes, and the rate for women with diabetes is 2.6 times that for women without diabetes. Cardiovascular disease (CVD) causes up to 65 % of all deaths in developed countries of people with diabetes. The World Health Organization (WHO) estimated that, in 2001, 959,000 deaths worldwide were caused by diabetes, accounting for 1.6 % of all deaths, and approximately 3 % of all deaths caused by noncommunicable diseases (21).

2.5 Pathophysiology of type 2 diabetes

2.5.1. Insulin resistance

2.5.1.1. Insulin hormone

Insulin is the hormone required to facilitate transport of blood glucose across the largely impermeable cell membrane. It is released in response to elevations in blood glucose. The insulin binds to a receptor on the cell membrane, allowing the entry into muscle and fat cells of glucose to form glycogen, fatty acids to generate triglycerides, and amino acids for protein synthesis. It is thus an anabolic hormone. It is a potent stimulator of growth factors, including insulin-growth factor 1 (IGF-1). It also inhibits catabolic processes such as the breakdown of glycogen and fat, and decreases gluconeogenesis (22).

Should there be any disturbances to the binding of insulin to the receptors, or of the receptor response to insulin, there will be reduced insulin activity, or insulin resistance. If insulin is not functioning, there is reduced glucose entry into the cells, which is detected by the pancreas as raised blood sugar (hyperglycemia). The pancreatic response is then to produce more insulin (hyperinsulinaemia), to compensate for the lack of cellular response to the hyperglycemia (22).

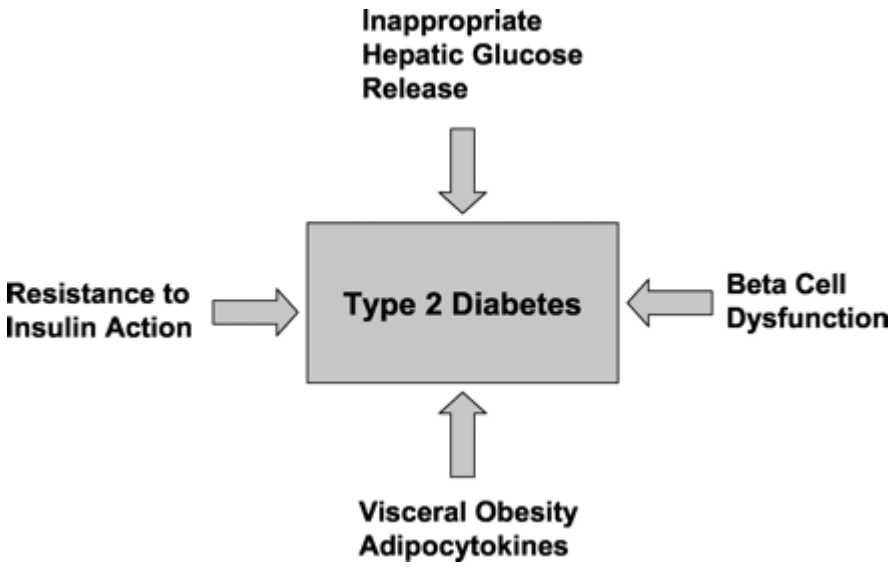
2.5.1.2 Evidences for the role of insulin resistance in T2DM

Although the primary factors causing T2DM are unknown, it is clear that insulin resistance plays a major role in its development. Evidence for this comes from:

a. the presence of insulin resistance 10–20 years before the onset of the disease,
b. cross-sectional studies demonstrating that insulin resistance is a consistent finding in patients with T2DM, and
c. prospective studies demonstrating that insulin resistance is the best predictor of whether or not an individual will later become diabetic (23).

2.5.1.3 Mechanisms of insulin resistance

In theory, insulin resistance may arise through a variety of mechanisms (Figure 2.1). Thus, it may result from defects in the insulin receptor gene, which encodes a transmembrane tyrosine kinase receptor, or as a consequence of post-receptor defects. Anti-insulin receptor antibodies that may arise in certain autoimmune disorders such as systemic lupus erythematosus can also block insulin receptor activation. In addition, certain hormones, e.g., cortisol, antagonize insulin action that may precipitate diabetes in susceptible individuals (24).



FIigure 2.1. Mechanisms of insulin resistance (adopted from reference number 24).

2.5.2 Pancreatic β-cell

It has been suggested that the final common pathway responsible for the development of T2DM is the failure of the pancreatic β-cell to compensate for insulin resistance (25). An absolute decompensation in β–cell function is seen during transition from IGT to diabetes. β-cell dysfunction results from preprogrammed genetic abnormalities, acquired defects, or a combination of both. First-phase or early insulin secretion following exposure to a glucose load prevents postprandial hyperglycemia. This acute glucose-induced insulin secretory response is lost early in the development of T2DM, while acute insulin responses to nonglucose stimuli such as arginine may by preserved. Eventually with further decompensation both phases of glucose-stimulated insulin secretion become impaired. Tight metabolic control of T2DM may improve first phase insulin secretion and normalize the proinsulin-to insulin ratio. However, the natural history of T2DM predicts progressive deterioration in β -cell function over time (7).

2.5.2.1 Mechanisms of β-cell function

The pancreatic β–cells secrete insulin in response to glucose stimulation through a series of transmembrane electrical reactions. Glucose metabolism in β-cells generates bursts of action potentials that ultimately lead to coupling of intracellular calcium influx with insulin secretion. The adenosine triphosphate (ATP) sensitive potassium channel (K-ATP) normally maintains the β-cell resting membrane potential, thereby preventing calcium entry. The K-ATP channel is inhibited when the ratio of ATP to adenosine diphosphate rises within the β-cell cytosol as occurs during glycolysis. The resultant depolarization of β-cell membrane activates calcium channels, and the intracellular calcium buildup triggers insulin secretion (7).

2.5.2.2 Mechanisms of β-Cell dysfunction

The molecular mechanisms of β-cell dysfunction remain to be fully clarified. A loss of β-cell differentiation can be found in the early stage of T2DM. Decreased gene expression of insulin-sensitive glucose transporter (GLUT-2), glucokinase, pyruvate carboxylase, transcription factors and increased gene expression of lactate dehydrogenase, hexokinase, glucose-6-phosphatase and transcription factor cellular myelocytomatosis (c-myc) have been described. The loss of β-cell mass is associated with deposition of amyloid, a product of human islet amyloid polypeptide normally produced in the β-cells and secreted along with insulin. Islet amyloid is a characteristic pathological finding in more than 90 percent of patients with T2DM. Patients who require insulin treatment have the greatest reduction in islet mass and the most prominent amyloid deposits. Islet amyloid deposition has been implicated in β-cell cytotoxicity through the expression of apoptosis-related genes, oxidative stress and/or formation of ion channels. The degree of islet amyloid formation correlates with the degree of hyperproinsulinemia. The demise of insulin secretory capacity that is linked to amyloid-induced cytotoxicity may be a target for therapies directed toward preservation of islet function in T2DM. Future therapies might be directed at inhibiting the production of human islet amyloid polypeptide or inhibiting amyloid fibrillogenesis (7).

2.5.3 Hepatic glucose output

Basal rates of hepatic glucose production (HGP) are variably increased in patients with T2DM but are normal in their normoglycemic first-degree relatives. The ability of insulin to suppress HGP also is normal in first-degree relatives, suggesting that dysregulated HGP is probably acquired late in the pathogenesis. In a recent prospective study of Pima Indians, endogenous glucose output remained normal during the transition from NGT to IGT, but increases by 15% with further progression to diabetes. Thus, in patients with established diabetes, the rate of hepatic gluconeogenesis is increased, and the endogenous glucose production becomes a key determinant of the fasting

plasma glucose. The increased endogenous glucose output is a result, at least in part, of hepatic insulin resistance. The diabetic state increases fat utilization as alternative fuel; products of the resultant lipolysis and fatty acid oxidation, such as acetyl-coenzyme A, are potent stimuli for hepatic gluconeogenesis. Glucagon levels also tend to be elevated in type 2 diabetic subjects. Glucagon stimulates the synthesis and release of glucose by the liver. Hepatic insulin resistance is associated with a decrease in glucokinase activity, which catalyzes a crucial step in hepatic glucose metabolism. The liver in T2DM thus appears to be programmed to overproduce and underutilize glucose. Clearly, hepatic glucose production is an important therapeutic target in T2DM (7).

2.5.4 Glucose toxicity

Hyperglycemia itself has detrimental effects on insulin secretion and on the action of insulin on peripheral tissues thus triggering a vicious cycle that perpetuates the hyperglycemia. The fact that tight metabolic control can improve insulin secretion as well as glucose transport in patients with T2DM indicates that glucose toxicity is a reversible syndrome. The hexosamine biosynthesis pathway functions as a nutrient-sensing pathway capable of desensitizing the glucose transport system to insulin in skeletal muscle *in vivo* (7). The level of activity of the rate-limiting enzyme in hexosamine synthesis, glutamine:fructose-6-phosphate amidotransferase, is correlated with glucose disposal rates in normal humans and transgenic mice. This enzyme diverts glucose from the glycolytic pathway at the level of fructose-6-phosphate, resulting in the production of glucosamine-6-phosphate and subsequently other hexosamine products. Exposure of muscle to glucosamine reduces insulin-stimulated glucose transport and GLUT-4 translocation (26). Transgenic mice that overexpress glutamine:fructose-6-phosphate amidotransferase are resistant to the effects of insulin on glucose uptake in muscle. The activity of glutamine: fructose-6-phosphate amidotransferase is upregulated in skeletal muscle of subjects with diabetes. However, it is unclear whether inhibition of glutamine:fructose-6-phosphate amidotransferase will be an effective strategy in the prevention or amelioration of glucose toxicity. Interestingly, any intervention

that improves glycemic control improves the manifestations of glucose toxicity (7).

2.6 Genetics of type 2 diabetes

T2DM is a heterogeneous disorder that may result from defects in one or more diverse molecular pathways (27). It is a classical example of multifactorial disorder, the etiology of T2DM combines both genetic and environmental factors (Figure 2.2) (28). Identification of the genetic components of T2DM is the most important area of diabetes research because elucidation of the diabetes genes (alleles) will influence all efforts toward a mechanistic understanding of the disease, its complications, and its treatment, cure, and prevention (27).

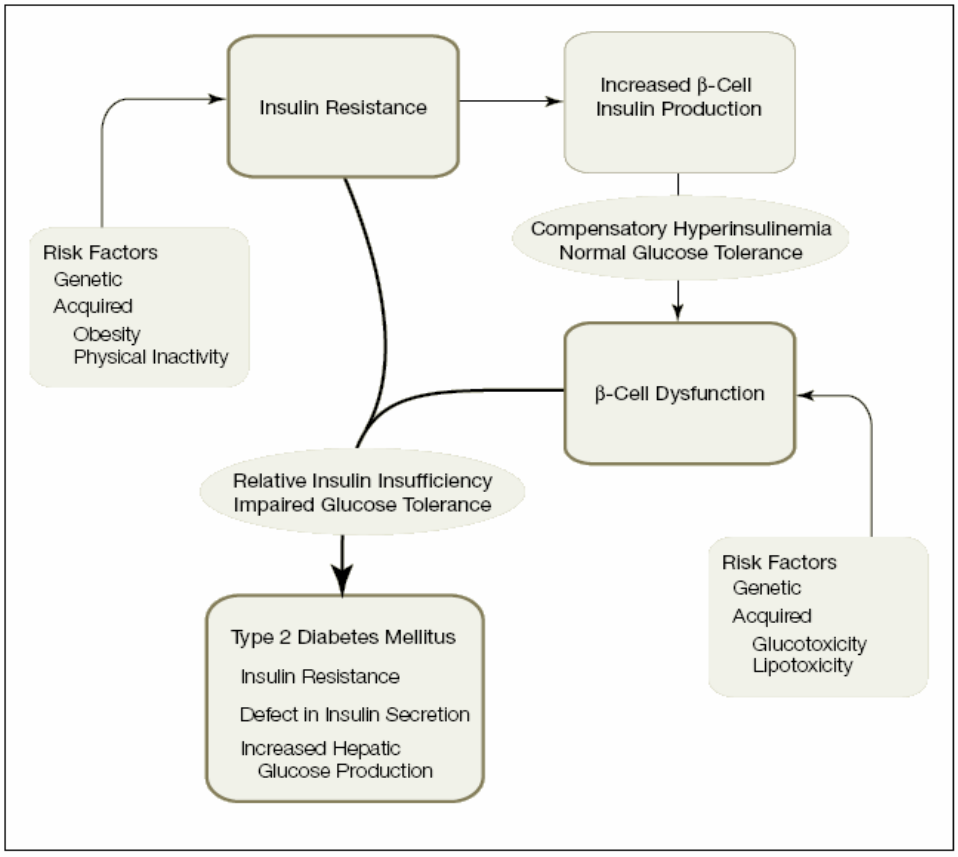


Figure 2.2. Schematic representation of the etiology of T2DM (adopted from reference number 19).

The genetic background of T2DM is undoubtedly heterogeneous. Most patients with T2DM exhibit two different defects: the impairment of insulin secretion and decreased insulin sensitivity (27). This means that there are at least two groups of T2DM susceptibility genes. The substantial contribution of genetic factors to the development of diabetes has been known for many years (29). The important pieces of evidence for the role of genes in T2DM are:

1) The observation of a wide spectrum of diabetes prevalence in different ethnic group's worldwide. Part of this observed ethnic variability can be attributed to non-genetic environmental and cultural factors; however, the observation that the disease prevalence varies substantially among ethnic groups that share similar environment (27), for example Pima Indians with extremely high prevalence of T2DM that can not be explained solely by environmental factors, supports the idea of genetic factors contributing to disease predisposition (29).

2) Apart form genes, families share environments, culture and habits, yet familial aggregation of the disease is another source of evidence for a genetic contribution to the disease. Different observations like the nearly 4-fold increased risk for T2DM in siblings of a diabetic proband compared with the general population, the increase in risk of T2DM in the offspring of one affected parent to an odds ratio (OR) of 3.4–3.5 and to 6.1 if both parents are affected etc. substantiate the role of genetic factors in T2DM etiology (27). It was shown that the level of insulin sensitivity in Caucasians is inherited and a low level is a poor prognostic factor that precedes the development of T2DM (29).

3) Twin studies suggesting that T2DM susceptibility can be explained by only a few genetic loci (oligogenic inheritance). Several studies of twin concordance rates have been undertaken in T2DM, and estimates for concordance rates ranged from 0.20 to 0.91 in monozygotic (MZ), while in dizygotic (DZ) the range was 0.10–0.43. In spite of several caveats in twin studies, the evidence is compelling that T2DM has a substantial genetic component (27).

4) Data from different laboratories supporting a genetic basis for insulin sensitivity and insulin secretion (27).

2.6.1 Genetic factors

Genetic factors play an important role in T2DM, but the pattern is complicated, since both impairment of β-cell function and an abnormal response to insulin are involved. Researchers have identified a number of genetic factors that may be responsible for selected or more general cases of diabetes (30):

- Researchers have identified genes responsible for maturity-onset diabetes in youth (MODY), a rare genetic form of T2DM that develops only in Caucasian teenagers
- A defective fatty-acid binding protein 2 (FABP2) gene may result in higher levels of unhealthy fat molecules (particularly triglycerides), which may be critical in the link between obesity and insulin resistance in some people with T2DM
- Alterations in five genes that influence β-cell and pancreas function have been identified that may plan an important role in inherited cases of T2DM
- Variations in a gene that regulates a protein called CAPN10 is proving to affect insulin secretion and action and may play a role in T2DM in certain populations
- Defective genes that regulate a molecule called peroxisome proliferator-activated receptor (PPAR) gamma may contribute to both T2DM and high blood pressure in some patients
- A defective gene has been detected that reduces activity of a protective substance called β_3-adrenergic receptor, which is found in visceral fat cells (those occurring around the abdominal region). The result is a slow-down in metabolism and an increase in obesity (30).

International genetic and genomic projects have delivered a wide range of tools and resources for genome-wide investigations to help the search for genetic

factors involved in T2DM and have contributed to recent progress in the knowledge of the etiology of the disease. Genetic markers and dense genetic maps allow extensive searches for gene variants that co-segregate with the disease. Although many genetic loci have been described that are linked to either diabetes or associated pathophysiological markers, CAPN10 remains the only known gene underlying polygenic T2DM (28).

2.6.2 Genome scans

For monogenic forms of T2DM, linkage analysis, recombinant mapping in families and positional cloning have proved to be powerful tools to define genomic T2DM regions, to further refine the location and ultimately to identify the gene encoding the disease. These findings have encouraged researchers to use genome-wide scans for linkage to identify the genetic basis for the more common forms of T2DM. The first published genome scan on T2DM on 170 Mexican American-affected sib-pair families showed significant evidence of linkage to T2DM near the terminus of 2q. Using linkage disequilibrium (LD) mapping, polymorphisms within the gene encoding *CAPN10* were found to explain the previous described linkage and to be associated with T2DM. Further analysis in Mexican American and European populations indicated that the disease susceptibility is best described by a combination of risk haplotypes (14).

2.7 Genetic forms of type 2 diabetes mellitus

As far as genetic background of T2DM is concerned, the disease may be divided into two large groups: monogenic and polygenic forms (Table 2.4)

2.7.1 Monogenic forms of T2DM

Monogenic forms are a consequence of rare mutations in a single gene. Mutations may affect the structure and subsequently the function of a protein or tRNA. In some cases they may be localized in regulatory parts of genes and alter gene expression. Monogenic forms are characterized by high phenotypic

Table 2.4. Genetic forms of T2DM

Monogenic	Polygenic, complex
1. Relatively rare, up to 10%	1. Frequent, about 90%
2. Consequence of rare, severe mutations	2. Consequence of frequent polymorphisms
3. Substantially impaired structure and function of protein or tRNA	3. Interaction of environment and some number of different genes
4. High phenotypic penetrance	4. Alleles of these polymorphisms are present, although with different frequencies in T2DM cases and controls
5. Early age of diagnosis	5. Late age of diagnosis, clinical picture usually less severe than in monogenic forms
6. Usually severe disease onset and clinical picture	
7. Critical role of genetic background, marginal role of environment	

Adopted from reference number 29.

penetrance, which means that the presence of the mutation practically determines the development of the disease. They are also characterized by early age of diagnosis, and frequently, but not always, a severe clinical picture, and occasionally the presence of extra-pancreatic features. Genetic background plays a critical role in their pathogenesis, while the environment only slightly modifies the clinical picture. The known forms of monogenic T2DM are characterized either by severe defect in insulin secretion or profound decrease in insulin sensitivity. Like in other Mendelian traits, in spite of their huge influence on the health of some individuals and families, their role in entire populations is very limited (29).

2.7.2 Multifactorial forms of T2DM

The clinical picture of complex T2DM, also called polygenic or multifactorial, is a result of the interaction between the environment and genetic background understood as the contribution of many different genes. Studies of patterns of inheritance indicate that probably multiple genes are involved. However the final number and the relative contributions of these genes are uncertain. Different loci may contribute to the development of T2DM. They may belong to the same or to different causal pathways. Some genes may contribute substantially to the development of diabetes in one population with no or very limited effect in another ethnic group. The susceptibility to complex forms of T2DM is associated with frequent polymorphisms that create amino acid variants in exons or influence the expression of genes in the regulatory parts. Alleles of those polymorphisms are present in both healthy individuals and in T2DM patients, although with different frequencies. Those sequence differences are associated with just a limited increase in the risk of developing the disease. So, they can be considered susceptibility variants, but not causative factors that unequivocally determine the disease. The diagnosis of polygenic T2DM usually occurs in the middle or late stage of life, and the level of glycemia rises steeply over the years (29).

2.7.3 Genetics of complex T2DM

As far as the search for genes responsible for the development of complex forms of diabetes is concerned, researchers are faced by several serious problems. Genetic heterogeneity, with different genes contributing to T2DM in different populations, the presence of different subtypes of T2DM which may be accounted for by different genes, population differences in linkage disequilibrium between markers and causal variants make the search for T2DM genes a real challenge. In recent years, hundreds of genes were examined for their potential role in the predisposition to T2DM. Out of many analyzed genes only a few of them were fully proven to influence the susceptibility to the disease (Table 2.5) (29).

Table 2.5. Genes with strong scientific evidence of association with complex forms of T2DM

Gene	Function	Polymorphism
Calpain 10	Protease	SNP43/19/63
PPAR	Transcription factor	Pro12Ala
Kir6.2	KATP channel subunit	Glu23Lys

Adopted from reference number (29).

2.8 Calpains

The calpains are a family of calcium-dependent, nonlysosomal cysteine proteases. They work by partial proteolysis, resulting in either activation or inhibition of substrate function and, as such, have been postulated to be an integral component of cell signaling (31). The presence of calpains in mammalian cells was first reported over 30 years ago. Since that time at least 14 members of the calpain family have been identified and their chemistry and biology have been extensively studied. Although its physiological function is still not fully understood, it is implicated in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation (32).

Molecular biological studies have shown that calpains constitute a superfamily, which exists ubiquitously in organisms ranging from microorganisms to humans. Calpain has attracted much attention because of the recent discovery of correlations between calpain gene mutations and human diseases (32). It has been proposed that alterations in calpain activity result in a number of disease states, including stroke, traumatic brain injury, Alzheimer's disease, cataracts, limb-girdle muscular dystrophy and gastric, T2DM and some types of cancer (Table 2.6). Several oncogenes and tumor-suppressor gene products are substrates for members of the calpain family (31, 33, 34).

Table 2.6. Examples of pathologic conditions that have been associated with the calpains

Disease	Observation
Limb-girdle muscular dystrophy type 2A	This disease is associated with mutations in the gene encoding calpain 3 (*CAPN3*) and probable loss of CAPN3 proteolytic activity.
Gastric cancer	This type of cancer is associated with down-regulation of *CAPN9.*
Type 2 Diabetes Mellitus	Mutations in intron 3 of *CAPN10* are associated with an increased incidence of T2DM in some populations.
Duchenne's and Becker's muscular dystrophies	These dystrophies are associated with the absence or deficiency of dystrophin, a membrane-associated protein, resulting in an increased Ca^{2+} level in muscle, loss of Ca^{2+} homeostasis, and inappropriate calpain activity.
Alzheimer's disease	There is an increased amount of m-calpain in the cytosolic but not the membranous fraction of the brain and in the neurofibrillary tangles of the brain.
Cataract formation	Ca^{2+} influx activates m-calpain, the predominant calpain in the lens, cleaving *a*- and *b*- crystallins but not *g*-crystallins; the crystallin fragments aggregate to form cataracts.
Myocardial infarction	Ca^{2+} homeostasis is lost in ischemic areas, triggering inappropriate calpain activity; desmin and *a* -spectrin are degraded in ischemic heart tissue by synthetic calpain inhibitors; protein and mRNA levels of m-calpain and μ-calpain increase after myocardial infarction.
Multiple sclerosis	Levels of the 150-kD calpain-specific degradation product of *a*-spectrin increase 50% in human multiple sclerosis plaques; degradation of the 68-kD neurofilament protein is inhibited by a synthetic calpain inhibitor.
Obsessive–compulsive disorder	Erythrocytes from patients with obsessive–compulsive disorder have significantly higher calpain activities than normal controls, a finding that could not be attributed to differences in memory function
Neuronal ischemia (stroke)	Calpastatin is degraded by calpain to a membrane-bound 50-kD polypeptide in ischemic brain tissue; calpains participate in both apoptosis and necrosis in tissue damage in ischemic areas.

Adopted from reference number (34).

2.8.1 Structure and properties of calpains

Calpain is not specific for certain amino acid residues or sequences but recognizes bonds between domains. As a consequence, calpain hydrolyzes substrate proteins in a limited manner, and large fragments retaining intact domains are produced by hydrolysis. Calpain is regarded as a bio-modulator, because properties of the substrate proteins are often modulated upon hydrolysis by calpain (32).

Two members of the calpain superfamily, calpains 1 (μ-calpain) and 2 (m-calpain), have been extensively studied. Each of these calpains, which differ in their sensitivity to Ca^{2+} (as the names indicate, μ- and m-calpains are activated by micro and milli-molar in vitro Ca^{2+} concentrations respectively) (35) consists of two different polypeptide subunits (34). The larger subunit (80 kD) has catalytic activity, whereas the smaller, 30-kD, subunit has a regulatory function. The large subunits of μ-calpain and m-calpain are encoded by the *CAPN1* and *CAPN2* genes, respectively (34). Calpains 3, 8a, 9, 11, 12, and 13 also have 80-kD and 30-kD subunits. The 80-kD subunit has four domains: domain I is important for regulating the activity and dissociation of the subunit; domain II, a catalytic domain, has two subdomains in the absence of Ca^{2+}; domain III binds Ca^{2+} and phospholipids; and domain IV, is important for dimer formation. The 30-kD regulatory subunit of calpains 1, 2, and 9 consists of two domains: domain V and domain VI, which is similar to domain IV of the catalytic subunit. The 80-kD subunit of the other calpains (3, 8a, 11, 12, and 13) do not interact with the 30-kD subunit, although they do have domain IV. Calpains 5, 6, 7, 8b, 10, and 15 are atypical calpains in that some of their domains have been deleted or replaced. They lack domain IV and therefore presumably do not associate with the 30-kD subunit (32, 34, 35).

2.8.2 Classification of calpains

There are two classes of calpains: one (comprising calpains 1, 2, 5, 7, 10, 13, and 15) is ubiquitous in cytosol; the other (comprising calpains 3, 6, 8, 9, 11, and 12) occurs only or mainly in certain tissues (32). For example, calpain 8 is

stomach-specific, and CAPN3 is largely specific to skeletal muscle, although it is also present in cardiac muscle and liver (36, 37). The amino acid sequence of skeletal-muscle CAPN3 is similar to the sequences of the ubiquitous μ-calpain and m-calpain (which are structural variants that catalyze the same reaction), but it contains three specific insertion sequences (34).

2.8.3 Mechanisms of calpain activation

The mechanisms by which calpains are activated and identify their protein targets are complex and poorly understood. A ubiquitous specific inhibitor, calpastatin, regulates calpain activity (38). The calpain–calpastatin interaction is important in regulation of the activity of μ-calpain and m-calpain, (37) but the nature of this regulation in living cells is not understood. Studies using calpastatin have shown that calpains are clearly involved in some types of apoptosis in specific cell types and in response to certain apoptotic signals (37).

An intricate strategy for the regulation of calpain activity seems necessary because calpain is an abundant cytoplasmic protease that can cleave many intracellular signaling and structural proteins. Membrane localization of calpains is an important mechanism for regulating their activity. In early work, it was thought that when a calpain binds to the plasma membrane, it is transformed from an inactive, proenzyme into an active, proteolytic enzyme by autolysis. However, other findings indicate that both μ-calpain and m-calpain are active proteolytic enzymes before autolysis and that interaction with a membrane may bind calpains to their substrates, rather than promote autolysis (34).

Once activated on the membrane, the calpain presumably diffuses into the cytosol and becomes resistant to the inhibitory action of calpastatin. Substrate proteins are digested by the activated calpain on the membrane or in the cytosol. According to Gil-Parrado et al. (39), calpain activity is regulated not only by calpastatin but also by differential intracellular localization. In contrast, dissociation of the subunits that constitute a calpain appears to be less critical to

its regulation. Ca^{2+} levels required to initiate autolysis are as high as or even slightly higher than the levels required for proteolytic activity and are much greater than the free Ca^{2+} levels in living cells. A solution to this paradox appeared when it was discovered that the presence of phospholipids, with phosphatidylinositol, lowered the Ca^{2+} levels required for autolysis of μ-calpain and m-calpain. Other studies have shown that autolysis of these two calpains is an intermolecular process, rather than an intramolecular process, as previously thought (34).

μ-Calpain and m-calpain have diverse functions. They catalyze the proteolysis of proteins involved in cytoskeletal remodeling, cell-cycle regulation, signal transduction, cell differentiation, apoptosis and necrosis, embryonic development, and vesicular trafficking. For this reason, calpain activity has to be tightly regulated both temporarily and spatially to be effective and limited in scope (34, 36).

2.9 Calpain 10

CAPN10 is the first T2DM susceptibility gene to be identified through a genome scan, with polymorphisms being associated with altered *CAPN10* expression (31). The highest expression of *CAPN10* mRNA is found in human heart, followed by the pancreas, brain, liver and kidney (31, 36).

2.9.1 Calpain 10 discovery

It took several years of intensive searching to pinpoint CAPN10 as a gene responsible for linkage of T2DM with a region on chromosome 2q in Mexican-American population. It was eventually established that the association exists between several polymorphisms of CAPN10, a gene coding for the protein from the large family of cytoplasmic proteases, and T2DM in the examined population. The risk of T2DM development was associated with a haplotype created by three polymorphisms: SNP-43, SNP-63 and del/ins-19, rather than

with a variant of a single polymorphism. All those polymorphisms are localized in introns, and thus do not influence the amino acid structure of the protein. The likely pathophysiological mechanism involves CAPN10 gene expression. The importance of CAPN10 in the T2DM pathogenesis is different in various populations. For example, in Mexican-Americans this gene seems to be responsible for about 40% of T2DM familial clustering, while in a British population this value is several times lower. The way CAPN10 influences T2DM risk is still uncertain. This protein participates in the breakdown of other proteins and thus, through its proteolytic function, it can modulate the activity of other enzymes, and also modify the apoptosis process. However, the molecular mechanism associated with CAPN10, through which the impaired glucose metabolism occurs, still awaits to be clarified. It likely involves both an increase in insulin resistance and insulin secretion impairment. The interesting direct confirmation of pathogenetic importance of calpains in T2DM pathogenesis is the occurrence of IGT in AIDS patients that were treated by proteases inhibitors (29).

2.9.2 Gene structure and isoforms

CAPN10 is located on chromosome 2q37 (Figure 2.3), consists of 15 exons spanning 31 kb (Figure 2.4). Analysis of human cDNA clones revealed a complex pattern of alternative splicing, generating proteins of 672, 544, 517, 513, 444, 274, 139 and 138 amino acids (Figure 2.5). Several isoforms do not have an intact protease domain, suggesting that CAPN10 may have diverse cellular functions. Analysis by reverse transcription Polymerase chain reaction (RT–PCR) indicated that CAPN10a mRNA is the most abundant isoform in the various tissues examined. CAPN10c and 10g transcripts are easy to detect in many tissues, including skeletal muscle and pancreatic islets, and CAPN10h mRNA is present at moderate levels in islets, but not in any other tissue tested. The other forms, CAPN10b, 10d, 10e and 10f, seem much less abundant (4).

The presence of CAPN10 mRNA in pancreatic islets, muscle, and liver, the three most important tissues that control blood glucose levels, suggests that

CAPN10 may regulate pathways that affect insulin secretion, insulin action, and hepatic glucose production, each of which is altered in patients with T2DM (40).

2.9.3 Calpain 10 and type 2 diabetes mellitus

Determining the genetic risk factors that increase susceptibility to T2DM will improve our understanding of the mechanism underlying this disorder and perhaps lead to better therapies. The association of the gene encoding *CAPN10* with T2DM was initially reported by Horikawa and associates (4). Among the known calpains, CAPN10 is atypical, containing a domain III–like structure in place of the usual domain IV. So far, eight splice variants have

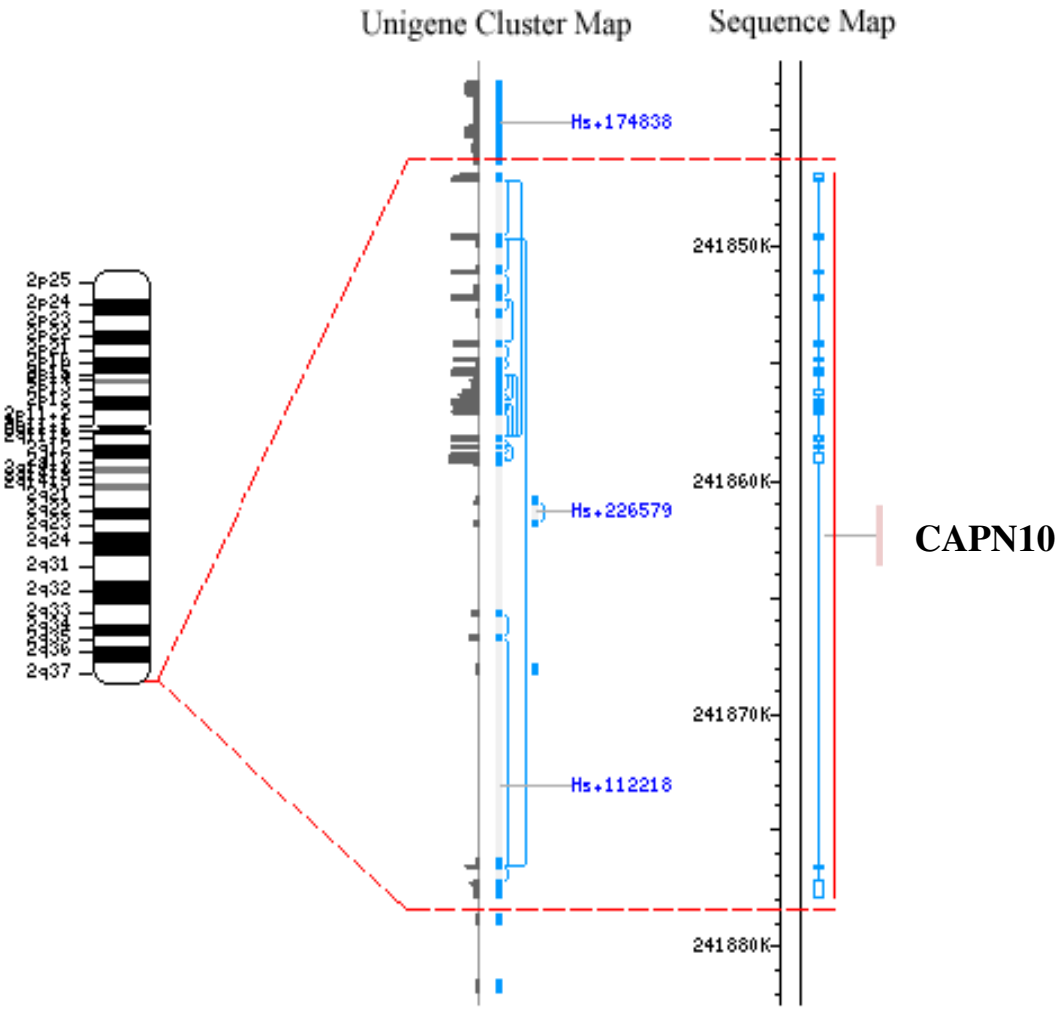


Figure 2.3. Location of CAPN10 on the human genome (adopted from reference number 5).

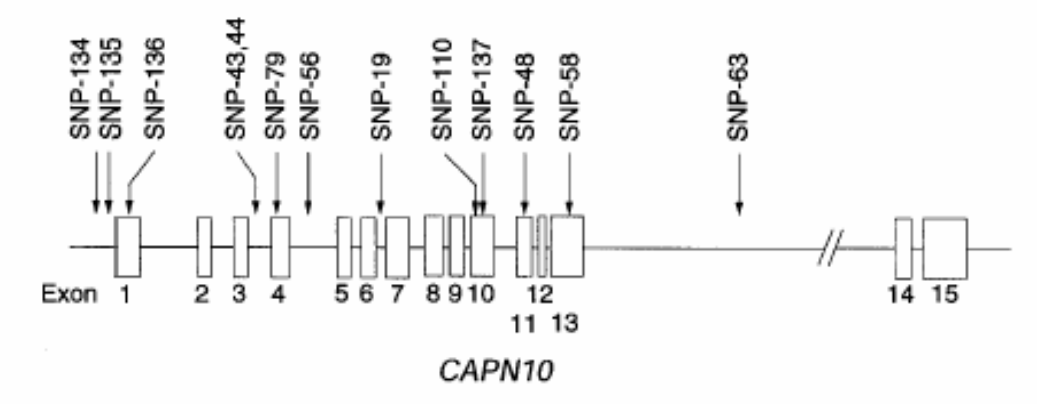


Figure 2.4 Exon-intron organization of *CAPN10* and the locations of the SNPs.

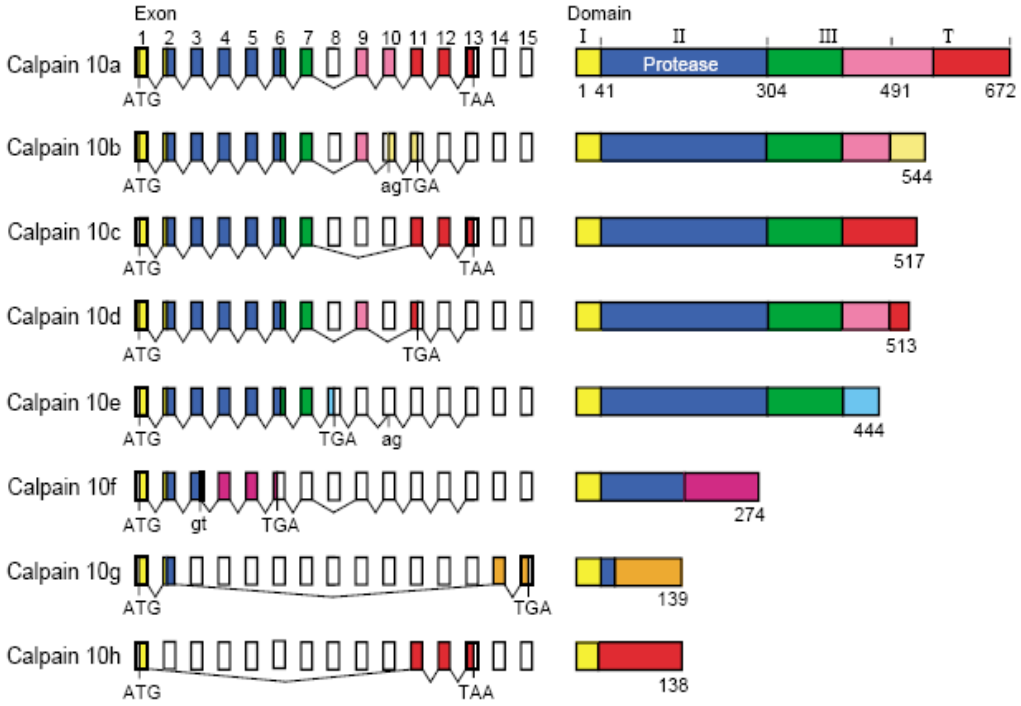


Figure 2.5 Alternative splicing of *CAPN10* mRNA generates a family of proteins. The patterns of alternative splicing and the organization of the CAPN10 proteins generated by alternative splicing are shown. The four domains that define calpains are noted. The numbers indicate the amino acid residue at the boundaries between domains. CAPN10 lacks domain IV and instead has a tandem (T) linking domain, domain III.

been identified, among them three with no protease activity. The CAPN10 protein is ubiquitous in adult and fetal human tissues. A polymorphism within intron 3 of *CAPN10* affects its translation to mRNA. Decreased levels of CAPN10 mRNA were observed in association with the G/G genotype, leading to up-regulation of protein kinase C activity. Since down-regulation of this kinase is important for proper phosphorylation of insulin receptors, this polymorphism could cause insulin resistance. Elevated FFA levels are also associated with some variants of *CAPN10.* CAPN10 may have a role in the actin reorganization that is required for insulin-stimulated translocation of insulin-responsive glucose transporter 4 to the plasma membrane in adipocytes, suggesting that there is a link between CAPN10 activity and T2DM (41).

2.9.4 Genetic variation in *CAPN10*

There is a complex relationship between susceptibility to T2DM and polymorphisms in *CAPN10*. Susceptibility is attributable not to a single polymorphism or allele, but rather to multiple polymorphisms whose collective effects are not easily predicted without having the full genotype/haplotype information at all contributing sites. If such allelic complexity is a feature of other complex traits, the identification of the genetic variation underlying the trait will be more difficult than if only a single polymorphism is involved (4). Genetic variation in CAPN10 seems to affect susceptibility to T2DM in both Mexican Americans and Europeans. The 112/121-haplotype combination (Figure 2.6) is associated with similar increased risk (threefold) of T2DM in both groups. Because the 112/121-haplotype combination is more common in Mexican Americans than Europeans, however, *CAPN10* has a much greater role in determining susceptibility in Mexican Americans than in Europeans. It is estimated that the 112/121-haplotype combination accounts for 14% of the population-attributable risk in Mexican Americans, whereas it only accounts for 4% in Europeans. The results of preliminary studies in the Pima Indians and in other European populations (British and French) suggest that genetic variation in *CAPN10* affects risk in these groups as well. The effects, however, are small and similar to those observed for the Finnish and German groups. Studies of

CAPN10 and T2DM are ongoing in other European groups as well as African Americans, Japanese and South Asians. The results of studies in other racial and ethnic groups may provide a better understanding of the relationship between genetic variation in *CAPN10* and the development of T2DM. They may also lead to the identification of other genetic variation at *CAPN10* that affects susceptibility (4).

Polymorphism	43	19	63
Allele base (number)	G(1)	3R(2)	C(1)
	G(1)	2R(1)	T(2)

Figure 2.6. Haplotype combination associated with the highest risk of T2DM in Mexican Americans: 43 is a G/A site polymorphism, 19 is a polymorphism with either two or three repeats of a 32-bp unit, and 63 is a C/T site polymorphism.

2.10 Overview of studies on CAPN10 in type 2 diabetes mellitus

Many of the studies on variation at *CAPN10* provide support for the general hypothesis that variation at *CAPN10* can affect susceptibility to T2DM and related phenotypes, studies on the high-risk haplotype combination and risk of T2DM have yielded variable results.

Horikawa et al. (2000) (4) found that 3 polymorphisms in CAPN10 defined a diabetes high-risk haplotype in Mexican Americans: SNP-43, del/ins-19, and SNP-63. The haplotype that was associated with greatest risk of diabetes in Mexican-Americans was also associated with the greatest risk in Finnish and German populations. It was concluded that genetic variation in CAPN10 is associated with T2DM (4). Horikawa et al. (2000) also reported that individuals with a combination of two different haplotypes (121/112) were at higher risk for T2DM, but found no evidence that individuals homozygous for either the 112 or the 121 haplotype were at high risk for T2DM.

In contrast, a number of studies in European populations, notably the studies of Orho-Melander et al. (42) in a Finnish population and of Malecki et al. (9) in a population from Poland, reported associations between T2DM and the homozygous 121 haplotype. Orho-Melander et al. observed increased frequencies of the G(1) allele at SNP-43 and the T(2) at SNP-63 in Finnish subjects with T2DM compared with control subjects. In addition, they reported a significantly increased risk for T2DM in individuals homozygous for the 121 haplotype (OR 1.93, 95% CI 1.07–3.47), and a similar risk for individuals with the high-risk haplotype combination (OR 1.85, CI 0.90–3.81). In the Malecki et al. study, individuals homozygous for the 121 haplotype had a significantly increased risk of T2DM (OR 1.93, CI 1.03–3.54). Individuals with the 121/112 haplotype combination found to be of high risk in Mexican Americans were not at increased risk of T2DM in this Polish population (OR 0.93, CI 0.39–2.23).

Elbein et al. (43) reported a significantly increased T2DM risk for individuals with the 111/221 haplotype combination at *CAPN10* (OR 1.48, CI 1.06–1.91) in their studies on a Utah population of European descent. Additional studies done by Baier et al. (44) on quantitative phenotypes in the unrelated members of the families studied indicated that homozygosity for the G allele at SNP-43 was associated with increased mean glucose. Variation at polymorphisms 19 and 63 showed association with a variety of the quantitative traits examined (polymorphism 19 with fasting insulin, 60-min glucose, insulin resistance by homeostasis model assessment, polymorphism 63 with total insulin area under the curve, 90-min insulin, and 2-h insulin) (43). Studies in Pima Indians on SNP-43 are also consistent with a role for variation at *CAPN10* in insulin sensitivity, with nondiabetic individuals homozygous for the G allele at SNP-43 found to have decreased rates of postabsorptive and insulin-stimulated glucose turnover that apparently result from decreased rates of glucose oxidation (44). Individuals homozygous for the G allele also showed significant alterations in nutrient partitioning, with preferential oxidation of fat when given a diet of mixed nutrient composition (44).

Horikawa et al. (45) also found no evidence for association of the variation at *CAPN10* defining the high-risk haplotype combination with T2DM in a Japanese population. In studies conducted within subsets of patients matched for age, sex, body mass index (BMI), and duration of disease, the high-risk haplotype combination was associated with higher levels of glucose-induced serum insulin at 60 min under a hyperglycemic clamp and with serum free fatty acid concentrations at the end point of a euglycemic clamp study. Hegele et al. (46) also found no significant association of the single polymorphism they examined at *CAPN10* (SNP-43) and T2DM in Oji-Cree, although the (OR 1.30, 95% CI 0.93–1.81) was similar to that observed for studies in African Americans (47), when also examining only SNP-43. Similarly, Rasmussen et al. (48) reported no significant association of the high-risk haplotype combination in a Danish population (OR 1.32, CI 0.60–2.89) and Tsai et al. (11) reported no significant association of the high-risk haplotype combination in a Samoan population (OR 1.42, CI 0.68–2.98). Kang et al. (49) found that a novel 111/121 diplotype in CAPN10 gene is associated with T2DM in the Korean population.

2.11 Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels

Orho-Melander et al. (2002) (42) investigated the role of *CAPN10* in T2DM in a Finnish population using a case-control and two different family-based approaches. SNP-43 genotypes 11 and 12 were associated with higher fasting serum insulin, higher FFA concentrations in normoglycemic control subjects and with higher fasting FFA levels in type 2 diabetic patients. Multiple regression analysis further suggested SNP-43 as an independent predictor of fasting FFA levels.

Carlsson et al. (2004) (50) reported that the variation in *CAPN10* SNP-43 was associated with elevated triglyceride levels in obese Swedish subjects. Subjects homozygous for the SNP-43 G allele had significantly elevated triglyceride

levels compared with subjects carrying the A allele [1.7 (1.1–2.4) *vs.* 1.4 (1.0–2.0); $P = 0.03$].

Garant et al. (2002) (51) found that there were no significant differences in subjects with or without diabetes in any of the cardiovascular risk factors (HDL and LDL cholesterol, triglycerides, and blood pressure) at baseline between the participants carrying at least one copy of the A allele and those homozygous for the G allele at baseline. They also examined changes in diabetes- and obesity related phenotypes in non-diabetic subjects over the 9 years of follow-up. There was no significant differences between SNP-43 genotypes with respect to changes in any of the obesity-, diabetes-, or cardiovascular related traits except for triglycerides, in which there were greater increases in the subjects homozygous for the G allele.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

- Absolute Isopropanol (Sigma, USA)
- Acetic acid (Sigma, USA).
- Agarose Molecular Biology grade (Promega, USA)
- DMSO (Dimethyl Sulphoxide) (Sigma, USA)
- DNA molecular weight marker 100 bp ladder (Promega, USA)
- DNA molecular weight marker 25 bp ladder (Promega, USA)
- DNAse, RNAse free Water (Promega, USA)
- dNTPs: (dATP, dCTP, dGTP and dTTP) (Promega, USA)
- EDTA disodium salt (Promega, USA)
- Ethanol 70% (Sigma, USA)
- Ethidium bromide (Promega, USA)
- $MgCl_2$ (Promega, USA)
- Tris base (hydroxymethyl aminomethane) (Promega, USA)

3.1.2 Reagent kits

- Cholestrol FS determination kit (Diasys, Germany)
- Glucose GOD FS determination kit (Diasys, Germany)
- PCR kits (Promega, USA)
- Triglycerides FS determination kit (Diasys, Germany)
- Wizard Genomic DNA purification Kit (Promega, USA)

3.1.3 PCR primers

Primers used in the study were purchased from Operon, Germany.

1- Primers for SNP-44 (52)

Common reverse primer (44.1) **5` -CTCATCCTCACCAAGTCAAGGC- 3`**

Forward primer, allele 1 'T' (44.2): **5` -CAGGGCGCTCACGCTTGCTAT- 3`**

Forward primer, allele 2 'C' (44.3):

5` -GTGGGCAGAGGACTGGTGGGCGCTCACGCTTGCTTC- 3`

2- Primers for SNP-43 (50)

Forward primer (43.1): **5` -GCTGGCTGGTGACATCAGTGC- 3`**

Reverse primer (43.2): **5` -ACCAAGTCAAGGCTTAGCCTCACCTTCATA- 3`**

3- Primers for del/ins-19 (52)

This insertion/ deletion polymorphism was amplified by:

Forward primer, allele 1 (19.1): **5` -GTTTGGTTCTCTTCAGCGTGGAG- 3`**

Reverse primer, allele 2 (19.2): **5` - CATGAACCCTGGCAGGGTCTAAG- 3`**

4- Primers for SNP-63 (52)

Forward primer (63.1): **5`-AAGGGGGGCCAGGGCCTGACGGGGGTGGCG- 3`**

Reverse primer (63.2): **5` -AGCACTCCCAGCTCCTGATC- 3`**

3.1.4 Enzymes

- HhaI Restriction enzyme (New England BioLabs)
- NdeI Restriction enzyme (New England BioLabs)
- Taq Polymerase (promega, USA)

3.1.5 Instruments and disposables

The following instruments and disposables were used in the present study:

- Centrifuge
- Computer
- Digital Camera
- Electronic Balance
- Electrophoresis Tank
- Freezer, Refrigerator
- Hoefer Short wave UV Light Table (Transilluminator)
- Incubator

- Micro Centrifuge
- Microfuge tubes - 1.5 mL capacity
- Microfuge tubes for PCR - thin wall 0.2 mL and 0.5 mL capacity
- Microwave Oven
- Pipettes (Ependorff)
- Power Supply (Bio-Rad)
- Spectrophotometer UV-Vis
- Thermocycler (Ependorff)
- Vortex Mixer
- Water Bath

3.2 Study population

The present study is a case-control study.

3.2.1 T2DM patients group

The blood samples tested in the present study were collected from 50 diagnosed T2DM patients. The samples were collected from patients attending the diabetic clinic at Al Remal Clinic.

3.2.2 Control group

The blood samples tested in the present study were collected from 50 normal persons with normal fasting blood glucose and negative family history of T2DM among first degree relatives.

3.2.3 Ethical considerations

An approval to carry out the study was obtained from the **Helsinki Committee-Gaza** (Appendix 1). Patients and controls gave their consent for participation in the study and all the information that were obtained were kept confidential.

3.3 Methods

3.3.1 Sample collection

Blood samples were collected from patients and controls in the morning after 14–16 hours fasting. Three milliliters of venous blood were withdrawn from antecubital vein and collected in EDTA Vacuette tubes. The samples collected were processed within 4 hours of collection for the patients and within 1 hour of collection in the case of controls (to prevent reduction in glucose concentration). The plasma was separated for triglyceride, cholesterol and glucose (for control samples only) by centrifugation at 3000 rpm for 10 minutes. The separated plasma was kept at –20^{o}C until used for determination of triglycerides, cholesterol and glucose. The whole EDTA blood was kept at -70 ^{o}C to be used for DNA isolation.

3.3.2 Triglycerides level determination

Triglycerides level were determined by using DiaSys kit (DiaSys Diagnostic Systems- Germany).

Principle

Triglycerides were determined after enzymatic splitting with lipoprotein lipase. The Indicator used in this assay was quinoneimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase.

Procedure

- The reagents and samples were brought to room temperature
- The blank, samples, standard and control tubes were prepared by mixing the components shown in Table 3.1

Table 3.1. Components and volumes for Triglycerides test

Component	Blank	Sample, standard or control
Distilled Water	10 µl	---
Reagent	1000 µl	1000 µl
Sample, standard or control	---	10 µl

- Tubes were mixed and incubated at 37°C for 10 minutes
- Absorbance was read within 60 min at wavelength 500 nm against reagent blank

Calculation

With standard or calibrator

$$\text{Triglycerides (mg/dl)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Std / Cal}} \text{ X Conc. Std/ Cal (mg/dl)}$$

- The results were analyzed according to the following reference range from the Diasys Kit: Desirable: < 200 mg/dl
  Borderline high: 200 - 400 mg/dl
  Elevated: >400 mg/dl

3.3.3. Cholesterol determination

Cholesterol level were determined by using DiaSys kit (DiaSys Diagnostic Systems- Germany).

Principle

Cholesterol was determined after enzymatic hydrolysis and oxidation. The colorimetric indicator used in this assay was quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase.

Procedure

- The reagents and samples were brought to room temperature
- The blank, samples, standard and control tubes were prepared by mixing the components shown in Table 3.2

Table 3.2. Components and volumes for Cholesterol test

Component	Blank	Sample, standard or control
Distilled Water	10 µl	---
Reagent	1000 µl	1000 µl
Sample, standard or control	---	10 µl

- Tubes were mixed and incubated at 37^{o}C for 10 minutes
- Absorbance was read within 60 min at wavelength 500 nm against reagent blank

Calculation

With standard or calibrator

$$\text{Cholesterol (mg/dl)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Std / Cal}} \text{ X Conc. Std/ Cal (mg/dl)}$$

- The results were analyzed according to the following reference range from the Diasys Kit

Desirable:	≤ 200 mg/dl
Borderline high risk:	200 - 240 mg/dl
High risk:	>240 mg/dl

3.3.4 Glucose determination

Glucose level were determined by using DiaSys kit (DiaSys Diagnostic Systems- Germany).

Principle

Glucose was determined after enzymatic oxidation by glucose oxidase. The colorimetric indicator used in this assay was quinoneimine, which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase.

Procedure

- The reagents and samples were brought to room temperature
- The blank, samples, standard and control tubes were prepared by mixing the components shown in (Table 3.3)

Table 3.3: Components and volumes for glucose test

Component	Blank	Sample, standard or control
Distilled Water	10 µl	---
Reagent	1000 µl	1000 µl
Sample, standard or control	---	10 µl

- Tubes were mixed and incubated at 37^{o}C for 10 minutes
- Absorbance was read within 60 min at wavelength 500 nm against reagent blank

Calculation

With standard or calibrator

$$\text{Glucose (mg/dl)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Std / Cal}} \text{ X Conc. Std/ Cal (mg/dl)}$$

- The results were analyzed according to the following reference range from the Diasys Kit

  Adults (fasting): 70 – 115 mg/dl

3.3.5 DNA extraction from whole blood

DNA was extracted from whole blood samples by using Wizard Genomic DNA Purification kit (Promega, USA).

Procedure

1- Three hundred microliters of whole blood were added to 900 µl of cell lysis solution in a 1.5 ml microcentrifuge tube. Tube was inverted 5-6 times to mix and then incubated at room temperature (RT) for 10 minutes to lyse RBCs.
2- Tube was centrifuged at 13000 rpm for 20 seconds at RT, then the supernatant was removed and discarded without disturbing the white pellet. The tube was then vortexed vigorously for 10-15 seconds until the white blood cells (WBCs) were resuspended.
3- Three hundred microliters of nuclei lysis solution were added to the tube containing the resuspended cells. The solution was pipetted 5-6 times to lyse the WBCs.
4- One and a half microliters of RNase solution were added to the nuclear lysate and the tube was mixed and then incubated at 37^{o}C for 15 minutes, and then the tube was cooled to RT.
5- One hundred microliters of protein precipitation solution were added to the nuclear lysate and then the tube was vortexed vigorously for 10-20 seconds. The tube was then centrifuged at 13000 rpm for 3 minutes at RT.
6- The supernatant were transferred to a 1.5 ml microcentrifuge tube containing 300 µl of RT Isopropanol. The tube was gently mixed by inversion until white thread-like strands were visible.
7- Tube was then centrifuged at 13000 rpm for 1 minute at RT. The DNA was then visible as small white pellet.

8- The supernatant was then decanted and 300 µl of RT 70% ethanol were added to the DNA. The tube was inverted several times to wash the DNA pellet. Then the tube was centrifuged at 13000 rpm for 1 minute.
9- The ethanol was aspirated, and the tube was left to dry for 10-15 minutes.
10- One hundred microliters of DNA rehydration solution were added to the DNA and the tube was incubated at 65^{o}C for 1 hour to rehydrate the DNA.
11- The DNA was stored at 2-8 ^{o}C.

Detection of the quantity and quality of the extracted DNA

Agarose gel electrophoresis

The quality of the isolated DNA was determined by running 5 µl of each DNA sample on 1.0% agarose gel stained with ethidium bromide, then the DNA sample was visualized on a short wave U.V. transilluminator.

Spectrophotometery

The optical density (O.D.) at 260 nm of diluted fractions of the isolated DNA samples was measured by a spectrophotometer and the DNA concentration was calculated by considering 1 $O.D._{(260\ nm)}$ = 50 µg/ml DNA and taking into account the dilution factor.

3.3.6 Genotyping

3.3.6.1 Mutagenically separated polymerase chain reaction (MS-PCR) for SNP-44

- PCR was carried out in a total volume of 25 µl, the reaction components were as described in Table 3.4.
- Microfuge tubes were then placed in a thermocycler and PCR amplification was started according to the program described in Table 3.5.

- PCR products were separated on 3.0% agarose gel and were visualized by ethidium bromide staining. Allele 1 (T) is 71 bp, and allele 2 (C) is 86 bp band.

Table 3.4. PCR reaction components for amplification of CAPN10 SNP-44

Reagents	Volume (µl)	Final concentration
PCR buffer	2.5	1 X
dNTPs	0.25	200µM
Common reverse primer (44.1)	1.5	1000 nmol
Forward primer (allele 1) (44.2)	1.0	1000 nmol
Forward primer (allele 2) (44.3)	1.0	67 nmol
Taq Polymerase	0.2	1 unit
$MgCl_2$	1.5	1.5 mM
DNA	1	40 ng
Nuclease free water	16.12	

Table 3.5. Thermocycler program for PCR amplification of CAPN10 SNP-44

No. of cycles	Temperature (^oC)	Time
1	96	12 min
35	94	30 Sec
	60	30 Sec
	72	30 Sec
1	72	10 min

3.3.6.2 Polymerase chain reaction (PCR) and restriction fragment length polymorphism for SNP-43

- PCR was carried out in a total volume of 25 µl, the reaction components were as described in Table 3.6.
- Microfuge tubes were then placed in a thermocycler and PCR amplification was started according to the program described in Table 3.7.
- PCR product was detected as 254 bp on 3% agarose gel.

- PCR products were digested with 10 u NdeI for 16 hours at 37^{O}C.
- The digested products were separated on 4.0% agarose gel and were visualized by ethidium bromide staining. Allele 1 (G) was detected as a 254 bp band, and allele 2 (A) was detected as a 223 bp and 31 bp bands.

Table 3.6. PCR reaction components for amplification of CAPN10 SNP-43

Reagents	Volume (µl)	Final concentration
PCR buffer	2.5	1 X
dNTPs	0.25	10 µM
Forward primer (43.1)	2.0	10 pmol
Reverse primer (43.2)	2.0	10 pmol
Taq Polymerase	0.1	0.5 unit
$MgCl_2$	1.5	1.5 mM
DNA	1	40 ng
Nuclease free water	15.62	

Table 3.7. Thermocycler program for PCR amplification of CAPN10 SNP-43

No. of cycles	Temperature (^{O}C)	Time
1	94	5 min
32	94	30 sec
	60	30 sec
	72	30 sec
1	72	10 min

3.3.6.3 Polymerase Chain Reaction (PCR) for del/ins-19

- PCR was carried out in a total volume of 25 µl, the reaction components were as described in Table 3.8.
- Microfuge tubes were then placed in a thermocycler and PCR amplification was started according to the program described in Table 3.9.

- The PCR products were separated on 3.0% agarose gel and were visualized by ethidium bromide staining. Allele 1 (2 repeats of 32 bp sequence) was detected as a 155 bp band, and allele 2 (3 repeats of 32 bp sequence) was detected as a 187 bp bands.

Table 3.8. PCR reaction components for amplification of CAPN10 del/ins-19

Reagents	Volume (µl)	Final concentration
PCR buffer	2.5	1 X
dNTPs	0.25	10 µM
Forward primer (19.1)	2.0	10 pmol
Reverse primer (19.2)	2.0	10 pmol
DMSO	1.25	5 %
Taq Polymerase	0.1	0.5 unit
$MgCl_2$	1.5	1.5 mM
DNA	1	40 ng
Nuclease free water	12.87	

Table 3.9. Thermocycler program for PCR amplification of CAPN10 del/ins-19

No. of cycles	Temperature (^oC)	Time
1	94	12 min
35	94	30 sec
	60	30 sec
	72	30 sec
1	72	10 min

3.3.6.4 Polymerase chain reaction (PCR) and restriction fragment length polymorphism for SNP-63

- PCR was carried out in a total volume of 25 µl, the reaction components were as described in Table 3.10.
- Microfuge tubes were then placed in a thermocycler and PCR amplification was started according to the program described in Table 3.11.
- PCR product was detected as 192 bp on 3% agarose gel.
- PCR products were digested with 2 u HhaI for 2 hours at $37^{O}C$.
- The digested products were separated on 3.0 % agarose gel and were visualized by ethidium bromide staining. Allele 1 (C) was detected as a 162 bp band, and allele 2 (T) was detected as a 192 bp band.

Table 3.10. PCR reaction components for amplification of CAPN10 SNP-63

Reagents	Volume (µ)	Final concentration
PCR buffer	2.5	1 X
dNTPs	0.25	10 µM
Forward primer	2.0	10 pmol
Reverse primer	2.0	10 pmol
DMSO	1.25	5 %
Taq Polymerase	0.1	0.5 unit
$MgCl_2$	1.5	1.5 mM
DNA	1	40 ng
Nuclease free water	14.37	

Table 3.11. Thermocycler program for PCR amplification of CAPN10 SNP-63

No. of cycles	Temperature (^{O}C)	Time
1	94	12 min
35	94	30 sec
	62	30 sec
	72	30 sec
1	72	10 min

3.3.7 Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 13 for Windows. For normally distributed data, means and standard deviations were calculated. Odds ratio and confidence intervals (95%) were reported where appropriate. Triglycerides concentrations were log transformed before analysis to achieve a normal distribution. Statistical significance was at the 5% level.

Chapter 4 Results

4.1 Characteristics of T2DM patients and control group

The characteristics of the patients and control group are presented in Table 4.1. The number of males and females in the patients group are 10 and 38 respectively, while in the control group their respective numbers were 12 and 36. The mean age in patients was 56 ± 9 years while in the control group the mean age was 41 ± 9 years. The mean for the duration of disease in the patients was 14 ± 10 years.

Table 4.1. **Characteristics of T2DM patients and control group**

	Patients	**Control**
Women/ men	38/10	36/12
Age (years)	56 ± 9	41 ± 9
Duration of disease (years)	14 ± 10	----

4.2 Genotyping results

4.2.1 Allele distribution of CAPN10 SNPs and del/ins-19

The allele distribution of CAPN10 SNP–44, SNP-43, del/ins-19, and SNP–63 were examined in the T2DM patients and control subjects (Table 4.2). The frequency of allele 1 “T” at SNP-44 was 74% in patients and 89% in controls (P=0.01), and the frequency of allele 1 “G” at SNP-43 was 84% in patients and 89% in controls (P=0.40). While the frequency of allele 1 “ 2 repeats 32 bp” at del/ins-19 was 43% in patients and 40% in controls (P=0.66), and the frequencies of allele 1 “C” at SNP-63 and allele 2 “T” were 93% and 7% for both patients and controls (P=1.00). The 2 X 2 contingency Chi square test was used to assess the allele frequency comparisons.

Table 4.2. Allele distribution of CAPN10 SNPs -44, -43, -63 and del/ins-19 in T2DM patients and the control group

		Allele 1	Allele 2	P-Value
SNP-44	**T2DM**	71 (74%)	25 (26%)	**0.01 ****
(A1=T, A2=C)	**Controls**	85 (89%)	11 (11%)	
SNP-43	**T2DM**	81 (84%)	15 (16%)	0.40
(A1=G, A2=A)	**Controls**	85 (89%)	11 (11%)	
Del/ins-19	**T2DM**	41 (43%)	55 (57%)	0.66
(A1=2 Rep., A2= 3 Rep.)	**Controls**	38 (40%)	58 (60%)	
SNP-63	**T2DM**	89 (93%)	7 (7%)	1.0
(A1=C, A2=T)	**Controls**	89 (93%)	7 (7%)	

A1= Allele 1, A2= Allele 2, Rep.= Repeats, ** statistically significant at the 0.01 level

4.2.2 Genotype distribution of CAPN10 SNPs and del/ins-19

The genotype distribution of CAPN10 SNP–44, SNP-43, del/ins-19, and SNP–63 were examined in the T2DM patients and control subjects (Table 4.3). There were no significant difference in genotype distribution for all the SNPs nor for del/ins-19. For SNP-44, the frequency of the T/T was 65% in patients vs 83% in the control subjects; and the T/C was 19% vs 10%; while the C/C was 16% vs 7%; p=0.1. For SNP-43, the frequency of the G/G was 69% in patients vs 77% in control subjects; and the G/A was 31% vs 23%; while the genotype A/A was not found neither in patients nor in controls; p=0.36. For del/ins-19 the frequency of the homozygous “2 repeats of 32 bp sequence” was 25% vs 23% in controls, and that of the heterozygous “2 repeats/ 3 repeats” was 35% in patients vs 33% in controls; while the homozygous for “3 repeats of 32 bp repeat” was 40% in patients vs 44% in controls; p=0.92. For SNP-63, the frequency of the C/C was 85% in both patients and controls, and the C/T was 15% in both patients and controls, while the genotype T/T was not found neither in patients nor in control subjects; p=1.00. The 2 X 2 and 2 X 3 contingency Chi square tests were used to assess the genotype frequency comparisons.

Table 4.3. Genotypes distribution of CAPN10 SNP-44, SNP-43, Del/ins-19 and SNP–63 in T2DM patients and control group

		Genotypes			
		1/1	**1/2**	**2/2**	**P-Value**
SNP-44	**T2DM**	31 (65%)	9 (19%)	8 (16%)	0.10
	Controls	40 (83%)	5 (10%)	3 (7%)	
SNP-43	**T2DM**	33 (69%)	15 (31%)	0	0.36
	Controls	37 (77%)	11 (23%)	0	
del/ins-19	**T2DM**	12 (25%)	17 (35%)	19 (40%)	0.92
	Controls	11 (23%)	16 (33%)	21 (44%)	
SNP-63	**T2DM**	41 (85%)	7 (15%)	0	1.00
	Controls	41 (85%)	7 (15%)	0	

SNP-43: genotype 1/1, G/G; genotype 1/2, G/A; genotype 2/2, A/A. Estimated haplotypes: SNP-43: allele 1, G; allele 2, A. SNP-19: allele 1, two repeats of 32-bp sequence; allele 2, three repeats of 32-bp sequence. SNP-63: allele 1, C; allele 2, T.

4.2.3 Genotype frequency and Hardy-Weinberg equilibrium

All genotypes frequencies were in Hardy-Weinberg (HW) equilibrium for both T2DM patients and the control group (Table 4.4) except for SNP-44, where both T2DM patients and the control group were not in HW equilibrium (p= 0.002). The Chi square goodness-of-fit test was used to assess deviation from HW equilibrium.

Table 4.4. Hardy-Weinberg test for patients and controls

	T2DM			**P(HW test)**	**Controls**			**P(HW test)**
Variant	**1/1**	**1/2**	**2/2**		**1/1**	**1/2**	**2/2**	
SNP-44	31	9	8	0.002	40	5	3	0.002
SNP-43	33	15	0	0.46	37	11	0	0.64
Del/ins-19	12	17	19	0.06	11	16	21	0.07
SNP-63	41	7	0	0.62	41	7	0	0.62

4.2.4 Distribution of CAPN10 SNPs -44, -43, -63 and del/ins-19 haplotypes

Analysis of the haplotype frequencies between the patients and controls has shown that no statistical significance was evident between the two groups (Table 4.5). The 2 X 2 contingency Chi square test was used to assess the haplotype frequency comparisons.

Table 4.5. Distribution of CAPN10 SNPs -44, -43, -63 and del/ins-19 haplotypes in T2DM patients and control group

	T2DM (n=48)	Controls (n=48)	P-Value	OR	CI
111	0.40	0.36	0.67	0.88	0.49 – 1.57
112	0.03	0.03	1.00	1.00	0.20 – 5.08
121	0.40	0.45	0.47	1.24	0.70 – 2.20
122	0.02	0.04	0.68	2.04	0.37 – 11.43
221	0.13	0.12	0.66	0.83	0.35 – 1.95
222	0.02	0			

4.2.5 Haplotype combinations of CAPN10 SNPs -44, -43, -63 and del/ins-19

The analysis of the results showed that there was no statistical significance between the two groups (Table 4.6). The 2 X 2 contingency Chi square test was used to assess the haplotype-combination frequencies between patients and control subjects.

Table 4.6. Distribution of CAPN10 SNPs -44, -43, -63 and del/ins-19 haplotypes combinations in T2DM patients and control group

	T2DM (n=48)	Controls (n=48)	P Value	OR	CI
111/111	0.19	0.17	0.79	0.87	0.30 – 2.48
111/112	0.06	0.06	1.00	1.00	0.19 – 5.22
111/121	0.17	0.17	1.00	1.00	0.34 – 2.93
111/122	0.04	0.06	1.00	1.53	0.25 – 9.61
111/221	0.10	0.10	1.00	1.00	0.27 – 3.71
121/121	0.23	0.29	0.49	1.39	0.55 – 3.46
121/221	0.17	0.13	0.56	0.71	0.23 – 2.24
Others	0.04	0.02			

4.3 Results of genotyping and triglycerides/ cholesterol levels

4.3.1 Comparison of triglycerides mean and cholesterol mean between T2DM patients and controls

Statistical analysis of the difference in triglycerides means of patients and controls showed significance difference ($p< 0.01$) (Table 4.7). The mean of triglycerides was higher in patients than in controls. The difference in cholesterol means of patients and controls also showed significant difference ($p=0.01$) (Table 4.7). The mean of cholesterol was higher in patients than in controls. t-student test was used to assess this relation.

Table 4.7. Comparison of triglycerides and cholesterol means between T2DM and controls

	T2DM	Control	P Value
Triglycerides (mg/dl)	227 ± 117	132 ± 71	< 0.01 **
Cholesterol (mg/dl)	199 ± 38	181 ± 29	0.01 **

** Statistically significant at the 0.01 level.

4.3.2 Comparison between the triglycerides and cholesterol levels and genotypes in patients and controls

Tables 4.8, 4.9, 4.10 and 4.11 represent comparisons between the triglycerides and cholesterol levels and genotypes in patients and controls in SNP-44, SNP-43, del/ins-19 and SNP-63.

There was no statistically significant difference in triglycerides levels and different genotypes in all SNPs nor in Del/ins-19 for both patients and controls. For SNP-43, there was no statistical significance in the difference in cholesterol levels and genotypes in both patients and controls. While for SNP-43, cholesterol levels in heterozygous patients was higher than in homozygous patients for the G allele ($p < 0.01$). For del/ins-19, cholesterol levels were the highest in patients having the homozygous allele 2 " 3 repeats of 32 bp". t-student test was used to assess the relation in SNP-43 and SNP-63, and ANOVA to assess the relation in SNP-44 and del/ins-19.

Table 4.8. Comparison between the triglycerides/ cholesterol levels and genotypes in T2DM patients and controls for SNP-44

		Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
T2DM	**1/1**	2.33 ± 0.29	0.87	198 ± 43	0.24
	1/2	2.29 ± 0.15		198 ± 43	
	2/2	2.27 ± 0.26		187 ± 26	
Controls	**1/1**	2.06 ± 0.20	0.63	183 ± 30	0.30
	1/2	2.18 ± 0.10		156 ± 13	
	2/2	2.17 ± 0.18		183 ± 15	

Table 4.9. Comparison between the triglycerides/ cholesterol levels and genotypes in T2DM patients and controls for SNP-43

		Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
T2DM	**1/1**	2.27 ± 0.20	0.22	189 ± 32	<0.01 **
	1/2	2.40 ± 0.36		220 ± 42	
	2/2	0		0	
Controls	**1/1**	2.07 ± 0.18	0.40	181 ± 27	0.90
	1/2	2.12 ± 0.26		182 ± 36	
	2/2	0		0	

** Statistically significant at the 0.01 level.

Table 4.10. Comparison between the triglycerides/ cholesterol levels and genotypes in T2DM patients and controls for del/ins-19

		Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
T2DM	**1/1**	2.26 ± 0.26	0.75	178 ± 31	0.02 *
	1/2	2.31 ± 0.20		195 ± 40	
	2/2	2.34 ± 0.32		215 ± 34	
Controls	**1/1**	2.08 ± 0.10	0.76	173 ± 27	0.19
	1/2	2.11 ± 0.16		192 ± 27	
	2/2	2.06 ± 0.26		178 ± 30	

* Statistically significant at the 0.05 level.

Table 4.11. Comparison between the triglycerides/ cholesterol levels and genotypes in T2DM patients and controls for SNP-63

		Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
T2DM	**1/1**	2.32 ± 0.26	0.63	197 ± 37	0.55
	1/2	2.27 ± 0.33		207 ± 47	
	2/2	0		0	
Controls	**1/1**	2.07 ± 0.21	0.39	181 ± 30	0.80
	1/2	2.14 ± 0.10		184 ± 23	
	2/2	0		0	

4.3.3 Haplotypes combination and triglycerides/ cholesterol levels in patients

The patients having the haplotypes combination 111/111 have the lowest cholesterol levels in comparison to other haplotypes combinations, though this result was not statistically significant. While the patients having 121/221 haplotypes combination have the highest cholesterol levels in comparison to other haplotypes combination (p=0.005) Table 4.12.

Table 4.12. Haplotypes combination and triglycerides and cholesterol levels in patients

Haplotypes combination	Frequency	Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
111/111	9	2.35 ± 0.15	0.65	177 ± 32	0.051
111/112	3	2.03 ± 0.40	0.06	182 ± 32	0.43
111/121	8	2.25 ± 0.12	0.50	181 ± 27	0.15
111/122	2	2.46 ± 0.18	0.44	212 ± 29	0.64
111/221	5	2.28 ± 0.33	0.81	193 ± 43	0.73
121/121	11	2.25 ± 0.19	0.43	203 ± 34	0.69
121/221	8	2.46 ± 0.43	0.28	232 ± 28	0.005**
Others	2				

Means of triglycerides/ cholesterol of each haplotypes combination were compared to the means of triglycerides/ cholesterol of other haplotypes combination. ** Statistically significant at the 0.01 level.

4.3.4 Haplotypes combination and triglycerides/ cholesterol levels in control group

The results showed that the control subjects having 111/121 haplotypes combination have the highest levels of cholesterol in comparison to other haplotypes combinations, p=0.04 (Table 4.13).

Table 4.13. Haplotypes combinations and triglycerides/ cholesterol levels in the control group

Haplotypes combination	Frequency	Log (Triglycerides)	P-Value	Cholesterol (mg/dl)	P-Value
111/111	8	2.05 ± 0.08	0.69	174 ± 29	0.41
111/112	3	2.14 ± 0.14	0.54	171 ± 24	0.52
111/121	8	2.05 ± 0.19	0.69	200 ± 30	0.04 *
111/122	3	2.15 ± 0.10	0.50	198 ± 20	0.31
111/221	5	2.16 ± 0.12	0.31	174 ± 18	0.55
121/121	14	2.04 ± 0.24	0.43	172 ± 22	0.19
121/221	6	2.09 ± 0.35	0.94	189 ± 47	0.66
Others	1				

* Statistically significant at the 0.05 level.

4.4 DNA extraction

The integrity of the DNA was evaluated by running 5 µl of the DNA extracts on 1% agarose gel, the gel was stained with ethidium bromide, and then visualized on an UV transilluminator.

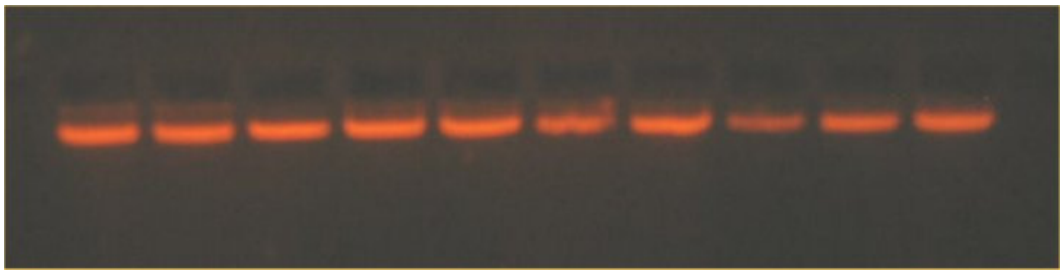


Figure 4.1. A Photograph representing the quality of 10 DNA samples extracted from whole blood samples, run on ethidium bromide stained 1% agarose gel.

4.5 PCR results

Figures 4.2, 4.3, 4.4, and 4.5 represent the PCR results for the genotyping of SNP-44, SNP-43, del/ins-19 and SNP-63, respectively.

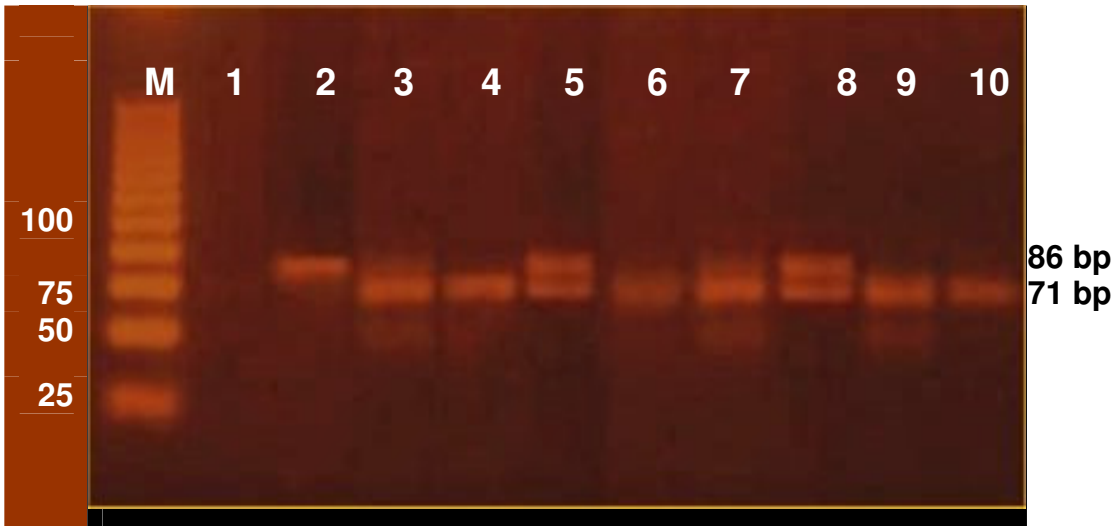


Figure 4.2. A photograph of ethidium bromide stained 3% agarose gel showing the MS-PCR product for SNP-44. M= 25 bp DNA ladder, lane 1 is a negative control; lane 2 indicates a homozygous sample for allele 2 (C/C); lanes 3,4,6,7 9 & 10 indicate homozygous samples for allele 1 (T/T); lanes 5 & 8 represent heterozygous samples (C/T).

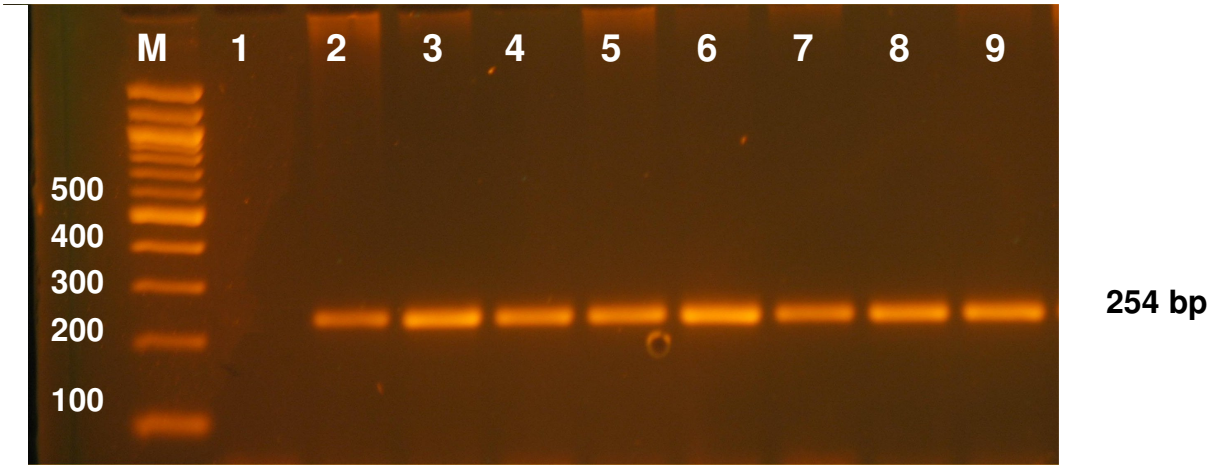


Figure 4.3. A photograph of ethidium bromide stained 3% agarose gel showing the PCR product of SNP-43. M=100 bp DNA ladder, lane 1 is a negative control; lanes 2-9 show the PCR product of SNP-43 (254 bp).

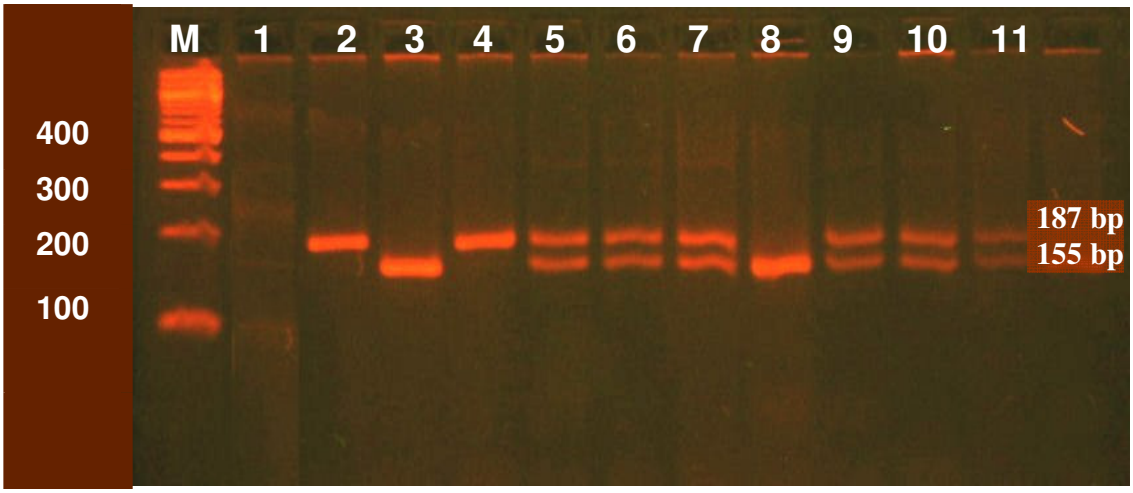


Figure 4.4. A photograph of ethidium bromide stained 3% agarose gel showing the PCR product of del/ins-19. M= 100 bp DNA ladder, lane 1 is negative control; lanes 2 & 4 show homozygous samples for allele 2 (3 repeats of 32 bp); lanes 3 & 8 show homozygous samples for allele 1 (2 repeats of 32 bp); lanes 5,6,7, 9, 10 & 11 show heterozygous samples (2 repeats32 bp/ 3 repeats 32 bp).

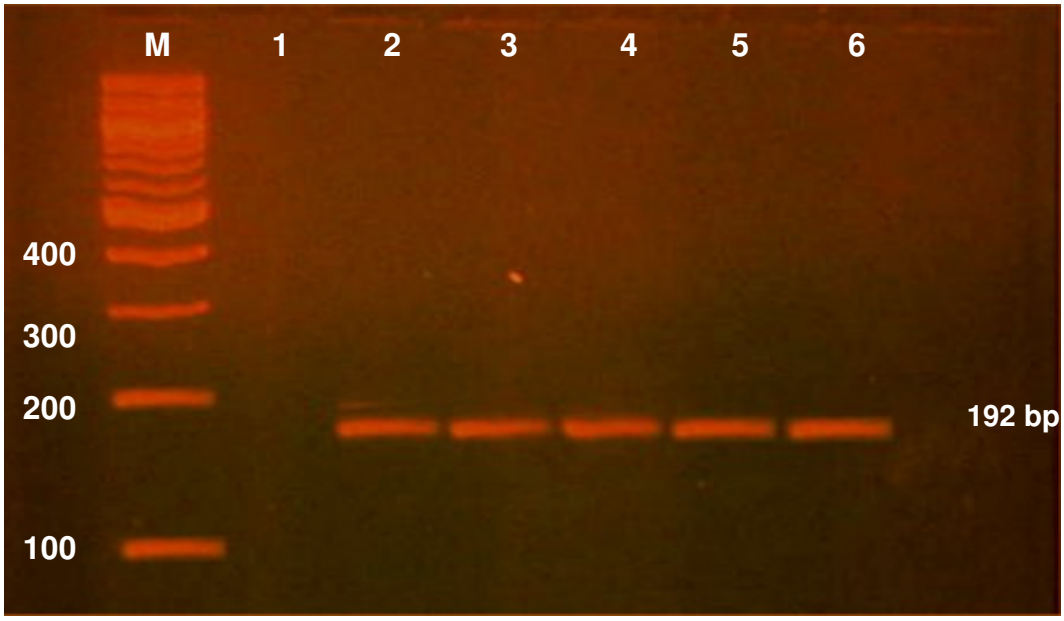


Figure 4.5. A photograph of ethidium bromide stained 3% agarose gel showing the PCR product of SNP-63. M= 100 bp DNA ladder, lane 1 is negative control; lanes 2 – 6 show the PCR product of SNP-63 (192 bp).

4.6 Restriction fragment length polymorphism (RFLP) results

This technique was used for the PCR products of SNP-43 and SNP-63, two restriction enzymes were used. NdeI restriction enzyme was used for SNP-43 product, allele G product was detected as a 254-bp fragment, allele A product was cut by the enzyme into 223- and 31-bp fragments. The second enzyme HhaI was used for SNP-63 product, allele T product was detected as 192 bp band, while allele C was cut by the enzyme into 162- and 30-bp fragments.

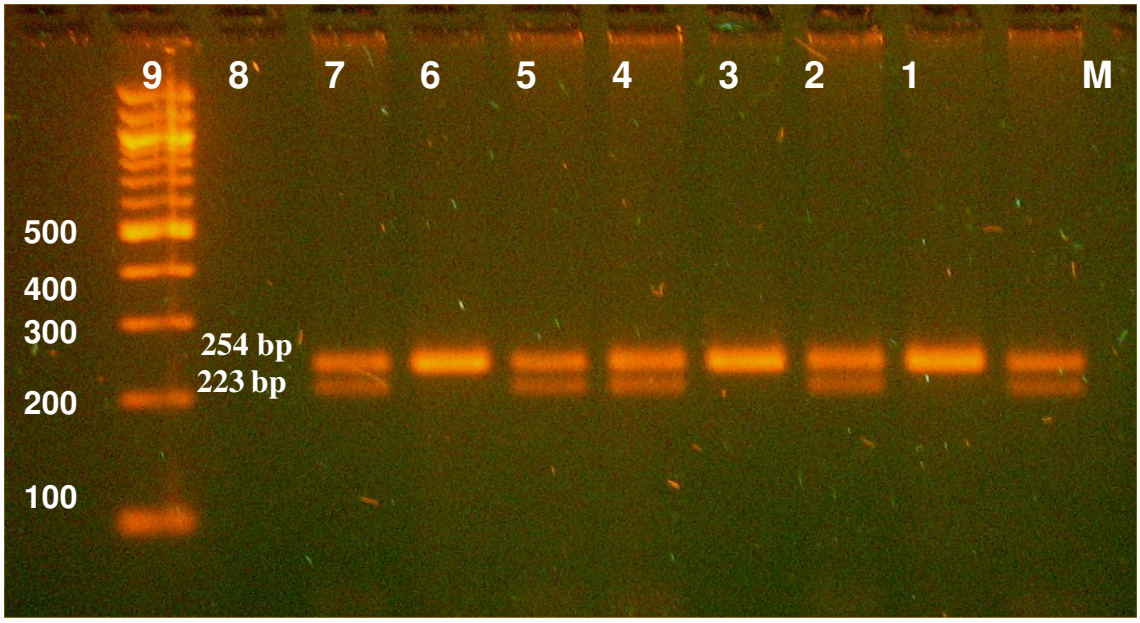


Figure 4.6. A photograph of PCR-RFLP products of SNP-43 run on ethidium bromide stained 4% agarose gel. M= 100 bp DNA ladder; lane 1 is a negative control; lanes 2, 4, 5 7 & 9 show digested and undigested products (heterozygous), lanes 3, 6 & 8 show undigested products (homozygous).

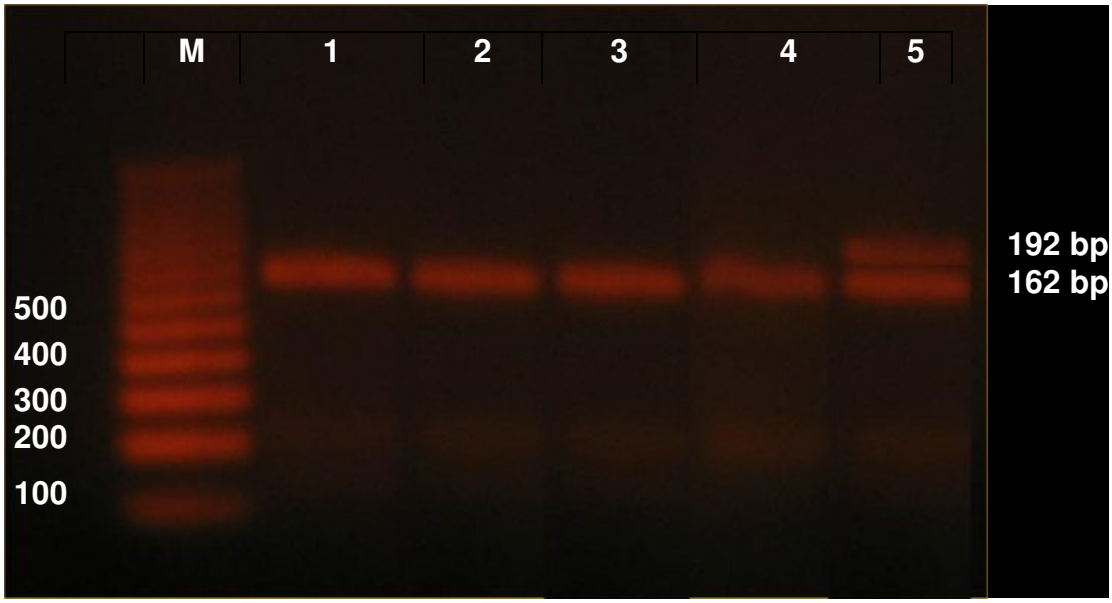


Figure 4.7. A photograph of PCR-RFLP products of SNP-63 run on ethidium bromide stained 4% agarose gel. M= 100 bp DNA ladder; lanes 1, 2, 3 & 4 show digested products (homozygous); lane 5 show digested and undigested products (heterozygous).

Chapter 5 Discussion

T2DM is a complex metabolic disorder which results primarily in a hyperglycemic condition. T2DM is a multifactorial disease in which environment triggers interact with genetic variants in the predisposition to the disease. It is believed that the study of the genetic factors that contribute to the development of diabetes will lead to improved diagnosis, treatment and prevention of this disorder, and thereby help to stem or even reverse the predicted rise in the prevalence of this disorder (4).

Calpain 10 is a member of a large family of intracellular proteases. It was the first candidate gene for T2DM identified through a genome-wide scan and positional cloning. Earlier studies on Mexican-Americans and other populations have shown that polymorphisms, SNP-44, SNP-43, del/ins-19 and SNP-63, of this ubiquitously expressed protein influence susceptibility to T2DM. However, substantial differences were shown between ethnic groups that are at risk and the alleles and haplotypes that they attribute. Thus, it is important to determine the role of CAPN10 in various populations.

Therefore, we have studied the effect that CAPN10 has on the risk of T2DM and on the levels of triglycerides and cholesterol in Gaza Strip. We have tested four polymorphisms in CAPN10, SNP-44, SNP-43, del/ins-19 and SNP-63 for association with T2DM, using case-control methods. We have selected these polymorphisms because of their prior association with either T2DM, either individually or in combination, or with the levels of triglycerides or cholesterol.

5.1 Association of variants in CAPN10 with T2DM

5.1.1 Association of allele frequencies of CAPN10 SNP-44, SNP-43, SNP-63 and del/ins-19 with T2DM in different ethnic groups

Table 5.1 below illustrates the allele frequencies of SNP-44, SNP-43, SNP-63 and del/ins-19 in the CAPN10 gene in several ethnic groups. The different allele frequencies observed in this study reflect the heterogenous genetic background of Gaza Strip population.

Table 5.1. Allele frequencies of SNPs -44, -43, -63 and del/ins-19 polymorphisms in the CAPN10 gene reported in 12 diverse populations.

	Frequency of							
	SNP-44 (T/C) T Allele		**SNP-43 (G/A) G Allele**		**SNP-19 (del/ins) del Allele**		**SNP-63 (C/T) C Allele**	
Study Population	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**
British/Irish	0.84	0.86	0.73	0.72	NA	NA	NA	NA
Finn	0.80	0.77	0.75	0.71	0.51	0.50	0.89	0.92
German	NA	NA	0.72	0.66	0.66	0.73	0.92	0.94
Polish	NA	NA	0.73	0.69	0.66	0.65	0.93	0.91
Scandinavian	NA	NA	0.73	0.72	0.62	0.60	NA	NA
African American	NA	NA	0.89	0.87	NA	NA	NA	NA
Mexican American	NA	NA	0.80	0.75	0.42	0.43	0.77	0.77
Samoan	NA	NA	0.91	0.91	0.67	0.63	0.85	0.84
Pima Indian	NA	NA	0.63	0.62	NA	NA	NA	NA
Chinese	0.87	0.91	0.90	0.89	0.70	0.67	0.80	0.78
Japanese	0.88	0.90	0.96	0.96	0.62	0.63	0.74	0.72
Gaza Strip	**0.74**	**0.89**	**0.84**	**0.89**	**0.43**	**0.40**	**0.93**	**0.93**

Modified from reference number (2). NA: not available.

Although the human chromosomes and the loci that they contain are identical throughout the species, the nature of different alleles and their frequencies at many loci vary widely among population groups. This can be due to selection,

mutation, or migration but usually occurs slowly, in small increments. Gene-gene or gene-environmental interactions could lead to the varying genetic effects of CAPN10 observed in different populations. For example, previous linkage analysis and a family-based test indicated an interaction between locus NIDDM1 on chromosome 2q37 and CYP19 on chromosome 15q21.1 in conferring increased susceptibility to T2DM (53).

According to the results of the current study, we did not find any association between T2DM and allele frequencies of SNP-43, del/ins-19, and SNP-63 individually; p-values were 0.40, 0.66 and 1.0, respectively. This result is different from that reported by Horikawa et al. (2000) (4), where the frequency of the common G-allele (allele 1) in SNP-43 showed a statistically significant increase in the patient group in the Mexican-Americans. In a Finnish population from Botnia, Orho-Melander et al. (2002) (42) found that SNP-43 allele 1 (G) and SNP-63 allele 2 (T) are associated with T2DM. In another study, Cassell et al. (2002) (54) found that SNP-63 is associated with T2DM in the South Indians.

Our result is compatible with those of Horikawa et al. (2003) (45), on Japanese, Tsai et al. (2001) (11), on Samoans, Evans et al. (2001) (52), on British; and those of Wu et al. (2005) (55) in their studies on Chinese where they all revealed no significant association between T2DM and allele frequencies of SNP-43, del/ins-19, and SNP-63 when tested individually.
We did, however, found significant difference in the allele frequency of SNP-44 (p=0.01), where the presence of allele 2 (C) was associated with a 2.7 fold increased risk of diabetes. Functional studies done by Horikawa et al. (2000) (4) suggested that SNP-44 is located in an enhancer element and might affect CAPN10 expression. In another study, a combined analysis of all United Kingdom studies, Evans et al. (2001) (52), found that the C-allele at SNP-44 was associated with a 1.5 – 2.5 fold increased risk of T2DM. Weedon et al. (2003) (56) also confirmed in a meta-analysis study that a CAPN10 haplotype defined by the SNP-44 polymorphism predisposes to T2DM.

The results of the current study, however, were not congruent with those of Jensen et al. (2006) (57), where they found no consistent evidence of association of the CAPN10 SNP-44 with T2DM.

5.1.2 Association of genotypes of CAPN10 SNP-44, SNP-43, SNP-63 and del/ins-19 with T2DM

The frequency of SNP-43 genotype 2/2 (allele A/A) was not found neither in T2DM patients nor in controls. This is different from frequencies found in the Europeans where the frequency was found to be 4-10%. On the other hand this genotype was not found in the Japanese neither in T2DM patients nor in controls (58, 59).

In the current study, we found that there is no significant difference in the genotypes distribution of the four studied variants (i.e, SNPs -44, -43, -63 and del/ins-19) with T2DM, p-values were 0.10, 0.36, 1.00 and 0.92, respectively (Table 4.3). Our result is compatible with that reported by Daimon et al. (2002) (58), where they found no difference in genotypes frequencies of SNP-44 and SNP-43. This result is also consistent with the results of Wu et al. (2005) (55), where no difference was observed in the distribution of genotypes of the four variants between patients and controls. The results are also compatible with those of Malecki et al. (2002) (9), Fingerlin et al. (2002) (60), and those of Kang et al. (2006) (49) where they all showed that there is no significant difference between T2DM and the different genotypes of the four variants.

Our result is different from that reported by Garant et al. (2002) (51), where they found significant association between homozygosity for the common G allele of SNP-43 of CAPN10 and typical T2DM in African-Americans.

5.1.3 Association of haplotypes of CAPN10 SNP-43, del/ins-19 and SNP-63 with T2DM

The haplotypes found in Gaza Strip are consistent with earlier reports which showed that only three to four of eight possible haplotypes occur in appreciable frequency in Europeans and other ethnic groups (59). Haplotype frequencies were 42.5% for 121, 38.0% for 111, 12.5% for 221, 3.0% for both 112 and 122, and 2.0% for 222. The most common haplotype in this study was 121, which is in agreement with other previous studies (Horikawa et al. 2000; Cassell et al. 2002; Fingerlin et al. 2002; Malecki et al. 2002; Wu et al. 2005) (4, 9, 54, 55, 60).

We found that there is no significant difference in the distribution of haplotypes among T2DM patients and control subjects. Our results are compatible with those of Fingerlin et al. (2002) (60), Evans et al. (2001) (52), Rasmussen et al. (2002) (48), Tsai et al. (2001) (11), Wu et al. (2005) (55) and also with those of Malecki et al. (2002) (9).

Our results are not, however, congruent with those of Kang et al. (2006) (49), where they found that the haplotype 111 showed a high risk of T2DM ($p<0.0001$).

5.1.4 Association of haplotypes combination of CAPN10 SNP-43, del/ins-19 and SNP-63 with T2DM

It has been demonstrated that the haplotype comprising the SNP-43, del/ins-19 and SNP-63 polymorphisms define risk of T2DM better than individual SNPs do; for example the haplotype combination 112/121 was found to be associated with increased risk of T2DM in Mexican Americans (4). We evaluated the frequency of the combinations comprising these alleles in the present study, and we found that neither the 112/121 haplotype combination nor the other haplotypes combinations has significant impact on increasing the risk of T2DM

in Gaza Strip. A similar study of Samoans (11) revealed no significant association between the different haplotype combinations. Our result is also compatible with that of Fingerlin et al. (2002) (60), and of Horikawa et al. (2003) (45).

On the other hand the results of the current study were not compatible with those of Orho-Melander et al. (2002) (42), where they found that the strongest risk haplotype combination in the Finnish population was the 1121/1121 (equivalent to 121/121 in our study). Cassell et al. (2002) (54) reported that the presence of the 1112/1121 (equivalent to 112/121 in our study) heterozygous haplotypes combination confers approximately 6.0 fold increased risk of T2DM in South Indians. Elbein et al. (2002) (43), found marginal evidence that the 111/221 haplotype combination increases the risk for T2DM after ascertainment correction in Caucasians. Malecki et al. (2002) (9), found that the 121/121 haplotype combination increases risk in Polish population. Kang et al. (2006) (49), found a significant association with the 111/121 haplotypes combination (odds ratio of 2.58). The high-risk 112/121 haplotypes combination for T2DM identified in Mexican Americans was not significant in the Korean population (49).

5.2 Association of variants in CAPN10 with triglycerides/ cholesterol levels

5.2.1 Correlation between the triglycerides/ cholesterol levels in T2DM patients and controls

The difference in means of triglycerides/ cholesterol levels of T2DM patients and controls showed significant difference ($p< 0.01$) and ($p=0.01$), respectively (Table 4.6). The means of triglycerides and cholesterol levels were higher in patients than in controls in both cases.

This result is consistent with the result of Kang et al. (2006) (49), where the level of triglycerides was shown to be higher in T2DM patients in comparison to controls (p=0.001). They also showed that cholesterol levels is higher in T2DM patients in comparison to controls, though this relation was not statistically significant. Garant et al (2002) (51) also showed that triglycerides levels are higher in T2DM patients than those in controls (p<0.001).

5.2.2 Association of CAPN10 genotypes SNP-44, SNP-43, SNP-63 and del/ins-19 with triglycerides/ cholesterol levels

Our results showed that there is association between the heterozygosity of SNP-43 in T2DM patients and cholesterol levels. T2DM patients with G/A genotype have higher cholesterol levels in comparison to those homozygous for allele 1 (G/G) (p<0.01). Our result is not consistent with those of Daimon et al. (2002) (58), where they showed that genotype combinations of SNP-43 G/G and SNP-44 T/T had significantly increased serum cholesterol levels (p=0.02).

We found that there is no association between SNP-43 and triglycerides level neither in patients nor in controls. This result is not consistent with the result of Carlsson et al. (2004) (50), where they found that obese non-diabetic Swedish subjects carrying the SNP-43 G/G genotype have significantly higher triglycerides level than subjects with the A/A allele. The result of Carlsson et al. is in agreement with earlier finding of Orho-Melander et al. (2002) (42), where they showed that fasting Free Fatty Acids (FFA) levels are significantly higher in carriers of the SNP-43 G allele compared with carriers of the A/A genotype.

Our results showed that T2DM patients homozygous for the del/ins-19 allele 2 (3 repeats of 32 bp) have cholesterol levels higher than those heterozygous or those which are homozygous for allele 1 (2 repeats of 32 bp). This result was not shown in other studies. This may be due to the limited number of studies which included triglycerides and cholesterol levels and their association to CAPN10.

5.2.3 Haplotypes combination and triglycerides/ cholesterol levels in T2DM patients

The results of the current study showed that T2DM patients having 121/221 haplotypes combination have higher cholesterol levels in comparison to other haplotypes combinations (p=0.005). This result also was not shown in other studies, the reason also may be assigned to the limited number of studies which tested the association between haplotypes combinations and triglycerides/ cholesterol levels in T2DM patients.

5.2.4 Haplotypes combination and triglycerides/ cholesterol levels in controls

Our results also showed that control subjects with haplotype combination 111/121 have higher serum cholesterol level than others without this haplotype combination. This result is different from that reported by Wu et al. (2005) (55), where they showed that the control subjects having the haplotypes combination 112/121 have higher cholesterol level than others without this haplotype combination in Chinese population.

5.2.5 Other genes that influence the susceptibility to T2DM

Genetic, functional genomic and transgenic studies have begun to uncover the molecular and cellular basis of insulin resistance, the hallmark of T2DM. Multiple groups have utilized genome-wide and candidate gene-based approaches to begin to identify the genetic underpinnings of T2DM. To date, several chromosomal regions, most notably chromosomes 1q21- q24, 2q37, 3q24- q27, 4q32- q33, 11q24, 12q and 20q have been identified as regions likely to harbor T2DM susceptibility genes. Furthermore, a few common variants in specific genes, each with modest effect, appears to be reproducibly associated with T2DM across several studies. These include calpain 10 (CAPN10), peroxisome proliferators-activated receptor-γ (PPARG), potassium

inward rectifying channel (KCNJ11) and perhaps common variants in the islet-specific promoter of HNF4α (HNF4A) (61).

5.3 Genetic prediction of future type 2 diabetes mellitus

The previous studies on diabetes that demonstrated the positive effects of strict blood glucose control led to a major clinical trial to prevent diabetes, the Diabetes Prevention Program (DPP). Study participants in the DPP were overweight (BMI over 25) and had blood glucose levels above normal but not at diabetic levels. The study demonstrated that modest weight loss (7% weight loss) and physical activity (150 minutes per week) can delay and even prevent the onset of diabetes in persons who demonstrate risk, but are not yet diagnosed. Just 30 minutes a day of moderate physical activity, coupled with a 5-10% weight loss, produced a 58% reduction in diabetes (62).

Lyssenko et al. (2005) (63) studied common variants in the *PPARG*, *CAPN10, KCNJ11, UCP2*, and *IRS1* genes for their ability to predict T2D in 2,293 individuals participating in the Botnia study in Finland. After a median follow-up of 6 years, 132 (6%) persons developed T2D. The hazard ratio for risk of developing T2DM was 1.7 (95% confidence interval [CI] 1.1–2.7) for the *PPARG* PP genotype, 1.5 (95% CI 1.0–2.2) for the *CAPN10* SNP-44 TT genotype, and 2.6 (95% CI 1.5–4.5) for the combination of *PPARG* and *CAPN10* risk genotypes. In individuals with fasting plasma glucose ≥5.6 mmol/l and body mass index ≥30 kg/m^2, the hazard ratio increased to 21.2 (95% CI 8.7–51.4) for the combination of the *PPARG* PP and *CAPN10* SNP43/44 GG/TT genotypes as compared to those with the low-risk genotypes with normal fasting plasma glucose and body mass index < 30 kg/m^2 (63).

It is important in case-control genetic studies that the size of the examined groups be large enough to detect a putative association. A study with more than 200 cases will be more robust than one with 50 subjects. We consider the small sample size used as a drawback in our study.

The results of our study are considered conclusive due to the following reasons:

- All the cases studied were confirmed T2DM patients at Al-Remal Clinic
- We were punctual in choosing the control subjects to be matched with cases regarding age (30-75 years), sex, and ethnic background. And also the glucose level was normal in controls, and a negative family history of T2DM among their first degree relatives
- Applied statistical tests were those that are commonly used in studies for case-control genetic association stratification

Although our sample size was not large enough, further studies on larger sample size will hopefully confirm our results.

Chapter 6 Conclusions & Recommendations

1. There is no association between T2DM and allele frequencies of SNP-43, del/ins-19 and SNP-63.

2. The presence of allele 2 (C-allele) in SNP-44 is associated with a 2.7 fold increased risk of T2DM

3. There is no significant difference in the distribution of genotypes, haplotypes and haplotypes combination, of the four studied variants, among T2DM and control subjects.

4. The levels of triglycerides and cholesterol were higher in T2DM patients than in control subjects.

5. There is association between the heterozygosity of SNP-43 in T2DM patients and cholesterol levels. T2DM patients with G/A genotype have higher cholesterol levels in comparison to those homozygous for allele 1 (G/G).

6. There is no association between the four studied variants genotypes and the level of triglycerides.

7. T2DM patients who are homozygous for del/ins-19 allele 2 (3 repeats of 32 bp) have higher cholesterol levels than those heterozygous or homozygous for allele 1 (2 repeats of 32 bp).

8. T2DM patients having the 121/221 haplotypes combination have higher plasma cholesterol level than those with other haplotypes combinations.

9. Control subjects with haplotypes combination 111/121 have higher plasma cholesterol level than those with other haplotypes combinations.

Recommendations

1. Use of a larger sample size to confirm our results.
2. Searching for correlation between T2DM and other genes e.g. PPARγ, KCNJ11, and HNF4A which were proved to be related to T2DM by other studies.
3. Investigation of the correlation between CAPN10 polymorphism and levels of other diabetic variables such as Insulin and glucose.
4. Study the correlation between CAPN10 polymorphism and other diseases such as Polycystic ovary syndrome.

Chapter 7 References

1. Wiltshire S., Hattersley A.T., Hitman G.A., Walker M., Levy J.C., and Sampson M. et al., (2001). A genomewide scan for loci predisposing to type 2 diabetes in a U.K. population (the diabetes UK Warren 2 Repository): analysis of 573 pedigrees provides independent replication of a susceptibility locus on chromosome 1q. *The American Journal of Human Genetics,* 69:553–569.
2. Song Y., Niu T., Manson J.E., Kwiatkowski D.J., and Liu S. et al., (2004). Are variants in the *CAPN10* gene related to risk of type 2 diabetes?. A quantitative assessment of population and family-based association studies. *The American Journal of Human Genetics,* 74:208–222.
3. Gavin J.R., Alberti K.G., Davidson M.B., DeFronzo R.A., Drash A., and Gabbe S.G. et al., (2002). Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care*, 25: S5-S20.
4. Horikawa Y., Oda N., Cox N.J., Li X., Orho-Melander M., and Hara M. et al., (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nature Genetics,* 26:163–175.
5. Dean L., and McEntyre J.R., (2004). *The genetic landscape of diabetes*. National Center for Biotechnology Information (NCBI), (pp. 1-2 to 1-5).
6. Cox N.J., (2001). Challenges in identifying genetic variation affecting susceptibility to type 2 diabetes: examples from studies of the calpain-10 gene. *Human Molecular Genetics*, 10(20): 2301-2305.
7. Moneva M.H., and Dagogo-Jack S., (2002). Multiple drug targets in the management of type 2 diabetes. *Current Drug Targets, 3:* 203-221.
8. Kahn B.B., and Flier J.S., (2000). Obesity and insulin resistance. *The Journal of Clinical Investestigation*. 106(4): 473-481.
9. Malecki M.T., Moczulski D.K., Klupa T., Wanic K., Cyganek K., and Frey J. et al., (2002). Homozygous combination of calpain 10 gene haplotypes is associated with type 2 diabetes mellitus in a Polish population. *European Journal of Endocrinology*, 146: 695–699.

10. Hanis C.L., Boerwinkle E., Chakraborty R., Ellsworth D.L., Concannon P., and Stirling B. et al., (1996). A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. *Nature Genetics,* 13(2): 161-166.
11. Tsai H.T., Sun G., Weeks D.E., Kaushal R., Wolujewicz M., and McGarvey S.T. et al., (2001). Type 2 diabetes and three c*alpain-10 g*ene polymorphisms in Samoans: no evidence of association. *The American Journal of Human Genetics,* 69: 1236–1244.
12. Lynn S., Evans J.C., White C., Frayling T.M., Hattersley A.T., and Turnbull D.M. et al., (2002). Variation in the Calpain-10 gene affects blood glucose levels in the British population. *Diabetes,* 51:247-250.
13. Khan N.M., and Hershey C.O., (2001). Update on screening for type 2 diabetes. Postgraduate Medicine, 109(2): 27-34.
14. Hansen T., (2002). Genetics of type 2 diabetes. *Current Science*, 83(12): 1477-1482.
15. Holt R.I.G., (2004). Diagnosis, epidemiology and pathogenesis of diabetes mellitus: an update for psychiatrists. *British Journal of Psychiatry*, 18(4): S55-S63.
16. Ministry of Health-Palestine Health Information Center (MOH-PHIC). Non communicable diseases. Health status in Palestine 2005, October 2006. http://www.moh.gov.ps/pdffiles/non-communicable-2005.pdf
17. Walker H.K., Hall W.D., and Hurst J.W., (1990). " The Endocrine System: Diabetes mellitus " In: *Clinical Methods*. (Butterworth Publishers, 3rd Edition), electronic book.
18. World Health Organization (1999) report of a WHO consultation. Part 1: Geneva: World Health Organization. http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf
19. Olefsky J.M., (2001). Prospects for research in diabetes mellitus. *The Journal of the American Medical Association*, 285(5): 628-632.
20. McCarthy M.I., (2004). Progress in defining the molecular basis of type 2 diabetes mellitus through susceptibility-gene identification. *Human Molecular Genetics*, 13(1): R33–R41.

21. Venkat Narayan K.M., Zhang P., and Kanaya A.M. et al., (2006). " Diabetes: the Pandemic and potential solutions " In: *Disease Control Priorities in Developing Countries*. (2nd edition) Chp.30: 591-593.
22. Cosford R., (1999). Insulin resistance, obesity and diabetes: the connection. *The Journal of the Australasian College of Nutritional and Environmental Medicine,* 18(1): 3-10.
23. Shulman G.I., (2000). Cellular mechanisms of insulin resistance. *The Journal of Clinical Investigation*, 106(2): 171-176.
24. Korc M., (2003). Diabetes mellitus in the era of proteomics. *Molecular & Cellular Proteomics, 2: 399-404.*
25. Polonsky K.S., Sturis J., and Bell G.I., (1996). Non-insulin-dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance. *The New England Journal of Medicine*, 334: 777-783.
26. Baron A.D., Zhu J.S., Zhu J.H., Weldon H., Maianu L., and Garvey W.T., (1995). Glucosamine induces insulin resistance in vivo by affecting GLUT 4 translocation in skeletal muscle. Implications for glucose toxicity. *The Journal of Clinical Investestigation*, 96(6): 2792–2801.
27. Das S.K., (2006). Genetic epidemiology of adult onset type 2 diabetes in asian indian population: past, present and future. *The International Journal of Human Genetics,* 6(1): 1-13.
28. Toye A., and Gauguier D., (2003). Genetics and functional genomics of type 2 diabetes mellitus. *Genome Biology*, 4(12): 241.1- 241.4.
29. Malecki M.T., and Klupa T., (2005). Type 2 diabetes mellitus: from genes to disease. *Pharmacological reports*, 57: S20-S32.
30. http://adam.about.com/reports/000060_1.htm
31. Marshall C., Hitman G.A., Partridge C.J, Clark A., Ma H., and Shearer T.R. et al., (2005). Evidence that an Isoform of calpain-10 Is a regulator of exocytosis in pancreatic ß-cells. *Molecular Endocrinology*, 19(1): 213-224.
32. Suzuki K., Hata S., Kawabata Y., and Sorimachi H. et al., (2004). Structure, activation, and biology of calpain. *DIABETES*, 53: S12–S18.

33. Otani K., Han D.H., Ford E.L., Garcia-Roves P.M., Ye H., and Horikawa Y. et al., (2004). Calpain system regulates muscle mass and glucose transporter GLUT4 turnover. *The Journal of Biological Chemistry*, 279(20): 20915–20920.
34. Zatz M., and Starling A., (2005). Mechanisms of disease: calpains and disease. *The New England Journal of Medicine*, 352: 2413-2423.
35. Sorimachi H., Ishiura S., and Suzuki K., (1997). Structure and physiological function of calpains. *Biochemistry Journal*, 328: 721-732.
36. Huang Y., and Wang K.K., (2001). The calpain family and human disease. *Trends in Molecular Medicine*, 7(8): 355-362.
37. Goll D.E., Thompson V.F., Li H., Wei W., and Cong J., (2003). The Calpain System. *Physiological Reviews*, 83: 731-801.
38. Ono Y., Kakinuma K., Torii F., Irie A., Nakagawa K., and Labeit S. et al., (2004). Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3. *Journal of Biological Chemistry*, 279(4): 2761-2771.
39. Gil-Parrado S., Popp O., Knoch T.A., Zahler S., Bestvater F., and Felgenträger M. et al., (2003). Subcellular localization and *in vivo* subunit interactions of ubiquitous μ-calpain. *Journal of Biological Chemistry*, 278(18): 16336-16346.
40. Sreenan S.K., Zhou Y.P., Otani K., Hansen P.A., Currie K.P.M., and Pan C.Y. et al., (2001). Calpains play a role in insulin secretion and action. *Diabetes,* 50: 2013-2020.
41. Johnson J.D., Han Z., Otani K., Ye H., Zhang Y., and Wu H. et al., (2004). RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. *The Journal of Biological Chemistry*, 279(23): 24794–24802.
42. Orho-Melander M., Klannemark M., Svensson M.K., Ridderstrale M., Lindgren C.M., and Groop L. et al., (2002). Variants in the calpain-10 gene predispose to insulin resistance and elevated Free fatty acid levels. *Diabetes*, 51: 2658-2664.
43. Elbein S.C., Chu W., Ren Q., Hemphill C., Schay J., and Cox N.J. et al., (2002). Role of calpain-10 gene variants in familial type 2 diabetes in

Caucasians. *The Journal of Clinical Endocrinology & Metabolism*, 87: 650–654.

44. Baier L.J., Permana P.A., Yang X., Pratley R.E., Hanson R.L., and Shen G.Q. et al., (2000). calpain-10 gene polymorphism is associated with reduced muscle mRNA levels and insulin resistance. *The Journal of Clinical Investestigation*, 106: R69–R73.
45. Horikawa Y., Oda N., Yu L., Imamura S., Fujiwara K., and Makino M. et al., (2003). Genetic variations in calpain-10 gene are not a major factor in the occurrence of type 2 diabetes in Japanese. *The Journal of Clinical Endocrinology & Metabolism*, 88: 244–247.
46. Hegele R.A., Harris S.B., Zinman B., Hanley A.J.G., and Cao H. et al., (2001). Absence of association of type 2 diabetes with *CAPN10* and *PC-1* polymorphisms in Oji-Cree. *Diabetes Care,* 24:1498-1499.
47. Fullerton S.M., Bartoszewicz A., Ybazeta G., Horikawa Y., Bell G.I., and Kidd K.K. et al., (2002). Geographic and haplotype structure of candidate type 2 diabetes susceptibility variants at the calpain-10 locus. *The American Journal of Human Genetics*, 70:1096–1106.
48. Rasmussen S.K., Urhammer S.A., Berglund L., Jensen J.N., Hansen L., and Echwald S.M. et al., (2002). Variants within the calpain-10 gene on chromosome 2q37 (NIDDM1) and relationships to type 2 diabetes, insulin resistance, and impaired acute insulin secretion among Scandinavian Caucasians. *Diabetes*, 51: 3561–3567.
49. Kang E.S., Kim H.J., Nam M., Nam C.M., Ahn C.W., and Cha B.S. et al., (2006). A novel 111/121 diplotype in the Calpain-10 gene is associated with type 2 diabetes. *The International Journal of Human Genetics*, 51: 629-633.
50. Carlsson E., Fredriksson J., Groop L., and Ridderstrale M. et al., (2004). Variation in the calpain-10 gene is associated with elevated triglyceride levels and reduced adipose tissue messenger ribonucleic acid expression in obese swedish subjects. *The Journal of Clinical Endocrinology & Metabolism*, 89(7): 3601-3605.

51. Garant M.J., Kao W.H., Brancati F., Coresh J., Rami T.M., and Hanis C.L. et al., (2002). SNP43 of CAPN10 and the risk of type 2 Diabetes in African-Americans: the atherosclerosis risk in communities study. *Diabetes*, 51: 231–237.
52. Evans J.C., Frayling T.M., Cassell P.G., Saker P.J., Hitman G.A., and alker M. et al., (2001). Studies of association between the gene for calpain-10 and type 2 diabetes mellitus in the United Kingdom. *The American Journal of Human Genetics*, 69: 544–552.
53. Engelman C., Barmada M., Ferrell R., and Norris J. et al., (2003). Investigation of an interaction between calpain-10 and CYP 19 in the susceptibility to type 2 diabetes. *Diabetes*, 52: A510–A511.
54. Cassell P.G., Jackson A.E., North B.V., Evans J.C., Syndercombe-Court D., and Phillips C. et al., (2002). Haplotype combinations of calpain 10 gene polymorphisms associate with increased risk of impaired glucose tolerance and type 2 diabetes in South Indians. *Diabetes*, 51: 1622–1628.
55. Wu B., Takahashi J., Fu M., Cheng H., Matsumura S., and Taniguchi H. et al., (2005). Variants of calpain-10 gene and its association with type 2 diabetes mellitus in a Chinese population. *Diabetes Research and Clinical Practice*, 68: 155-161.
56. Weedon M.N., Schwarz P.E., Horikawa Y., Iwasaki N., Illig T., and Holle R. et al., (2003). Meta-analysis and a large association study confirm a role for calpain-10 variation in type 2 diabetes susceptibility. *The American Journal of Human Genetics*, 73:1208–1212.
57. Jensen D.P., Urhammer S.A., Eiberg H., Borch-Johnsen K., Jorgensen T., and Hansen T. (2006). Variation in CAPN10 in relation to type 2 diabetes, obesity and quantitative metabolic traits: studies in 6018 whites. *Molecular Genetics and Metabolism*, 89: 360-367.
58. Daimon M., Oizumi T., Saitoh T., Kameda W., Yamaguchi H., and Ohnuma H. et al., (2002). Calpain 10 gene polymorphisms are related, not to type 2 diabetes, but to increased serum cholesterol in Japanese. *Diabetes Research and Clinical Practice*, 56: 147-152.

59. Tsuchiya T., Schwarz P., Bosque-Plata L., Geoffrey Hayes M., Dina C., and Froguel P. et al., (2006). Association of the calpain-10 gene with type 2 diabetes in Europeans: Results of pooled and meta-analysis. *Molecular Genetics and Metabolism*, 89:174-84.
60. Fingerlin T.E., Erdos M.R., Watanabe R.M., Wiles K.R., Stringham H.M., and Mohlke K.L. et al., (2002). Variation in three single nucleotide polymorphisms in the calpain-10 gene not associated with type 2 diabetes in a large Finnish cohort. *Diabetes*, 51:1644–1648.
61. Shuldiner A.R., and McLenithan J.C., (2004). Genes and pathophysiology of type 2 diabetes: more than just the Randle cycle all over again. *The Journal of Clinical Investestigation*, 114:1414–1417.
62. http://ndep.nih.gov/diabetes/prev/prevention.htm
63. Lyssenko V., Almgren P., Anevski D., Orho-Melander M., Sjögren M., and Saloranta C. et al., (2005). Genetic prediction of future type 2 diabetes. *Public Library of Science- Medicine*, 2: 1299-1308.

بسم الله الرحمن الرحيم

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Date: 14/6/2005 | **التاريخ: 2005/6/14**

Mr./ Mazen El Zaharna | السيد: مازن الزهارنة

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

Calpain 10 gene polymorphism in Type 2 Diabetes Mellitus patients in Gaza Strip.

In its meeting on June 2005
and decided the Following:-
To approve the above mention research study.

و ذلك في جلستها المنعقدة لشهر يونيو 2005
و قد قررت ما يلي:-
الموافقة على البحث المذكور عاليه.
على أن يتم الحصول على الموافقة الخطية من المرضى المراد إجراء البحث عليهم وتوضيحاً لهم لغرض البحث العلمي وأن يتم سحب الدم بواسطة شخص مختص بذلك

Signature
توقيع

Member	Member	Chairperson
عضو	عضو	

Ministry of Health
Helsinki Committee

Conditions:-

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.

Gaza Etwam – Telefax 972-7-2878166