APOLIPOPROTEIN E POLYMORPHISM AND ITS ASSOCIATION WITH BIOCHEMICAL MARKERS OF DIABETES MELLITUS

BY

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A thesis submitted in fulfilment of the requirements for the degree of Master in Pharmaceutical Sciences (Pharmacology)

Kulliyyah of Pharmacy
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SEPTEMBER 2013

ABSTRACT

Apolipoprotein E gene (APOE) has been known for more than 30 years and has been widely studied around the world for its role in the pathogenesis of diseases that are closely related to lipid and lipoprotein metabolism. Studies regarding the association between the APOE gene and type 2 diabetes mellitus (T2DM) are scarce. Therefore, this case-control study was aimed to investigate APOE allelic frequencies among the diabetic and non-diabetic subjects and associations between the alleles and selected bio-chemical markers between them. A total of 102 subjects were recruited, 51 were diabetic, and 51 were non-diabetic. Their fasting blood samples were analyzed for fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL). Restriction Fragment Length Polymorphism technique was used to identify the APOE alleles. Statistical analyses were performed using the Predictive Analytics SoftWare (PASW) Statistics, Version 18.0. Frequencies of the ε2 and ε4 alleles were slightly higher, and the ε3 allele was slightly lower in the diabetic group compared to the non-diabetic group (12.7% vs 8.8%, 24.5% vs 22.6%, and 62.8% vs 68.6% respectively). Diabetics with ε2 allele had the highest mean values of all selected bio-chemical markers, and the trend was followed by ε4 and ε3 alleles. Both the ε2 and ε4 allelic diabetic subjects had significantly higher FBG compared to the ε3 allelic diabetic subjects (10.11 vs 8.18 and 9.89 vs 8.18 mmol/L respectively, $p<0.05$). In contrast, diabetic subjects with ε2 and ε4 alleles had significantly higher TC (6.57 vs 4.58 and 5.41 vs 4.07 mmol/L respectively, $p<0.05$) while only ε4 allelic diabetic subjects had significantly higher TG (1.57 vs 0.97 mmol/L, $p<0.05$) compared to the non-diabetic subjects. In conclusion, the APOE gene polymorphism influences blood levels of the selected bio-chemical markers in subjects with T2DM.

ملخص البحث

البروتين الدهني (APOE)قد عرف منذ أكثر من 30 عاما وقد درس بشكل واسع حول العالم لدوره في التسبب في الأمراض التي ترتبط ارتباطا وثيقا بعملية التمثيل الغذائي للدهون والبروتين الدهني .ان الدراسات بشأن العلاقة بين جين APOE و مرض السكري من النوع الثاني (T2DM) نادره. ولذلك، فأن هذه الدراسة (دراسه الحالات والشواهد) كانت تهدف للتحقيق في تكرار أليلية APOE بين الماليزيين المصابين بمرض السكري و الماليزيين الغير مصابين بمرض السكري والعلاقة المشتركه بين الأليلات و والعلامات البيوكيميائية المختاره بينهما. مائة واثنان شخص تم شمولهم في هذه الدراسة, حيث كان واحد وخمسون شخص منهم مريض بالسكري و واحد وخمسون شخص ليس مصاب بالسكري. عينات دم المرضى (في حالة الصيام) تم تحليلها لقياس تركيز السكر في الدم(FBG), الكولسترول الكلي(TC), الدهون الثلاثية(TG), البروتين الدهني عالي الكثافة (HDL)و البروتين الدهني واطيء الكثافة(LDL). تم استخدام تقنية حصر الطول الجزئي للاشكال المتعددة (Restriction Fragment Length Polymorphism technique) للتعرف على أليلات البروتين الدهني (APOE alleles). لقد تم اجراء التحليلات الاحصائية باستخدام برنامج التحليل التنبؤي (PASW) الاصدار الثامن عشر. ان تكرارات الأليلات من نوع ε2 و ε4 كانت عالية قليلا و أليلات ε3 كانت واطئ قليلا في مجموعة مرضى السكري مقارنة مع مجموعة الغير مصابين بمرض السكري (12.7% مقابل 8.8%, 24.5% مقابل 22.6%, و 62.8% مقابل 68.6% على التوالي). ان مرضى السكري مع أليل ε2 كان لهم قيم متوسط عالية في العلامات البيوكيميائية المختاره, واعقب هذا الاتجاه أليلات ε4 و ε3. كان FBG عالي بشكل ملحوظ في مرضى السكري ذو الأليلات ε2 و ε4 مقارنة مع مرضى السكري ذو الأليلε3 (10.11 مقابل 8.18 و 9.89 مقابل 8.18 مل مول / ليتر على التوالي, $p<0.05$). في المقابل, مرضى السكري ذو الأليل ε2 وε4 كان لديهم TC عالي بشكل ملحوظ (6.57 مقابل 4.58 و 5.41 مقابل 4.07 مل مول / ليتر على التوالي, $P< 0.05$) بينما مرضى السكري ذو الأليل ε4 فقط كان لديهم TG عالي بشكل ملحوظ (1.57 مقابل 0.97 مل مول/ ليتر $P<0.05$) مقارنة مع الاشخاص الغير مصابين بمرض السكري. نستنتج من ذلك, ان متعدد الاشكال جين ال APOE يؤثر على مستويات الدم للعلامات البيوكيميائية المختارة في مرضى السكري من النوع الثاني T2DM.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmacology).

Tariq b. Abdul Razak
Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmacology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously of concurrently submitted as a whole for other degrees at IIUM or other institutions.

K. M. Hafizur Rahman

Signature……. Date……..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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Affirmed by K. M. Hafizur Rahman

……..……..…… ……..
Signature Date

This thesis is dedicated to

My Parents and Grandparents

It is a great pleasure for me to fulfil their dreams

ACKNOWLEDGEMENT

First of all, I would like to express my deepest gratitude to my supervisor, Prof. Dato' Dr. Tariq b. Abdul Razak for his valuable suggestions, painstaking supervision and constructive feedback. His smiling face, every time when I meet him, is a very special gift to me.

Special thanks must go to Dr. Seikh Farid Uddin Akter, Associate Professor of Kulliyyah of Medicine for introducing the International Islamic University Malaysia (IIUM) to me. He also provided a great shelter for me from the very beginning at the Non-Communicable Diseases Research Unit (NCD_RU). I also like to thanks Dr. Ahmad Marzuki Omar (Assistant Professor of Medicine, IIUM), who extended his helping hand during the study subject recruitment for this research. The helps of the other supporting staffs of the NCD_RU are not easily forgettable.

My sincere appreciation goes to Dr. Sarina Sulong (Pusat Genome Munasia, University Sains Malaysia), and Dr. Mohd Arifin Bin Kaderi (Assistant Professor of Kulliyyah of Allied Health Science, IIUM) for their careful and proper direction in learning molecular techniques from the very outset. I am thankful to all laboratory technicians and staffs of the Molecular Research Laboratory and Biochemistry Laboratory of Kulliyyah of Medicine for their kind assistance and countless services during my laboratory works. Here, I must acknowledge all laboratory staffs Kulliyyah of Pharmacy for their helping attitude with their limited resources.

Last but not least, it is very difficult to find proper words for me to express my gratitude to Him, who made me from a drop of blood and allowed me to play in the ocean of knowledge made by Him, ALLAH, the most merciful and the passionate. He not only provided me with such a great environment for this research but also let me be healthy and patient enough to complete this work.

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LIST OF ABBREVIATIONS / SYMBOLS

A_1C	Hemoglobin A_1C
ABCA1	ATP-binding-cassette-A1
AD	Alzheimer's Disease
ADA	American Diabetes Association
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analyses of variances
apo	Apolipoprotein in plasma
APOA	ApolipoproteinA gene
apoA	Plasma apolipoproteinA
apoB	Plasma apolipoproteinB
APOC	ApolipoproteinC gene
apoC	Plasma apolipoproteinC
APOE	ApolipoproteinE gene
apoE	Plasma apolipoproteinE
BP	Blood Pressure
cDNA	Cloned DNA
CHD	Coronary Heart Diseases
CVA	Cerebro-vascular accident
DCCT	Diabetes Control and Complications Trial
DDW	De-ionized distilled water
DHBS	3,5-Dichloro-2-Hydroxybenzenesulfonic Acid
DM	Diabetes Mellitus
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
DW	Distilled water
EtBr	Ethidium Bromide
FBG	Fasting Blood Glucose
FFA	Free Fatty Acids
GDM	Gestational Diabetes Mellitus
GK	Glycerol Kinase
GOX	Glucose Oxidase
GPO	Glycerol Phosphate Oxidase
H_2O	Water
H_2O_2	Hydrogen Peroxide
HBA	Hydroxyl benzoic acid
HDL	High Density Lipoprotein
HLP	Hyperlipidemia
IIUM	International Islamic University Malaysia
IRSR	Insulin receptor gene
Kb	Kilo-base
LADA	Latent adult onset autoimmune diabetes
LCAT	Lecithin:cholesterol-acyl-transferase
LDL	Low Density Lipoprotein
LDLr	LDL Receptor

LIPE	Hormone sensitive lipase gene
LPL	Lipoprotein Lipase enzyme
LRP3	LDLr–related–protein–type–3
$MgCl_2$	Magnesium Chloride
MODY	Maturity onset diabetes of the young
mRNA	Messenger RNA
NCD_RU	Non-Communicable Diseases Research Unit
NGSP	National Glycohemoglobin Standardization Program
NHMS	National Health and Morbidity Survey
O_2	Oxygen
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PASW	Predictive Analyses SoftWare
PCR	Polymerase Chain Reaction
POD	Peroxidase
QC	Quality Control
SNPs	Single Nucleotide Polymorphisms
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Serum Total Cholesterol
TG	Triglyceride
Tm	Melting temperature
UV	Ultra-violet
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
df	Degrees of freedom
p-	Power of significance
α	Alpha
β	Beta
δ	Delta
ε	Epsilon (APOE allele)
χ^2	Chi-squared test

CHAPTER ONE

INTRODUCTION

Most diseases in adulthood result from complex interactions between genetic factors and environmental factors. Disorders with a genetic basis are categorized as multi-factorial, chromosomal, or single gene disorders. Environmental factors can affect the phenotypic expression of the genetic defect (Walden and Hagele, 1994). Diabetes Mellitus (DM) is one of them. It is a potentially serious debilitating and deadly disease that has now reached epidemic proportion around the world and is on the increase (Mafauzy, 2006). Individuals diagnosed with type 2 diabetes mellitus (T2DM) have a two-fold to four-fold higher risk of developing coronary heart disease (CHD) than non-diabetics. In addition, approximately 60% of patients with diabetes die from CHD, and 50% develops peripheral vascular disease and its complications. Apolipoprotein E (APOE) gene has been suggested to be a risk factor for the development of micro- and macro-vascular complications in diabetic patients a decade ago (Shcherbak, 2001). Till now, there is no strong evidence to show an association between the APOE and T2DM. Certain studies described relationship with the complications caused by T2DM only. Thus, the APOE gene is an important candidate gene for the development of vascular complications in diabetic patients.

1.1 DIABETES MELLITUS

The term diabetes mellitus 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 (Consultation, 1999).

1.1.1 Classification

The classification of diabetes mellitus includes four clinical classes: (1) type 1 diabetes mellitus (T1DM), results from β-cell destruction, usually leading to absolute insulin deficiency, (2) type 2 diabetes mellitus, results from a progressive insulin secretory defect on the background of insulin resistance, (3) other specific types of diabetes mellitus due to other causes, e.g., genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical induced diabetes (such as in the treatment of AIDS or after organ transplantation), and (4) gestational diabetes mellitus (GDM), diabetes diagnosed during pregnancy (Care, 2010).

The most common types of diabetes mellitus observed in a primary care practice are T2DM and GDM. GDM is similar to T2DM, but is first diagnosed during pregnancy (Haas, 1998). T2DM is the most common form of diabetes mellitus constituting 90% of the diabetic population (Modak, Dixit, Londhe, Ghaskadbi and Devasagayam, 2007) and now found in almost every population (Alberti, Zimmet and Shaw, 2007). The prevalence is increasing with the population and their age, and the disease is present in one in every seven Malaysians (Azmi, 2012).

1.1.2 Epidemiology

Type 2 diabetes mellitus, by far the most common form of DM, is increasing at an alarming rate all over the world. Current projections estimate that the absolute number of cases worldwide may double over the next two decades (Bonora, Kiechl,

Willeit, Oberhollenzer, Egger, Meigs, Bonadonna and Muggeo, 2004). The prevalence of DM for all age groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with DM was projected to rise from 171 million in 2000 to 366 million in 2030 (Figure 1.1). The most significant rise was predicted in Asia and Africa; more precisely 164% in the Middle East, 162% in Africa, 161% in South East Asia, and in India was 150%. Latin America and Caribbean area then following them, 148% was predicted. China also showed a steep rise (104%) but not as high as mentioned by others before. Main factors behind this kind of rise would be high population growth rate, rapid changes of life style, food habits and types of occupation. The United States and Canada, and Australia also sowed rise above the expectation, 72% and 89% respectively, most probably due to migration from Asian communities. The lowest estimation was for Europe, which was only 32%; reduced population growth might be a cause.

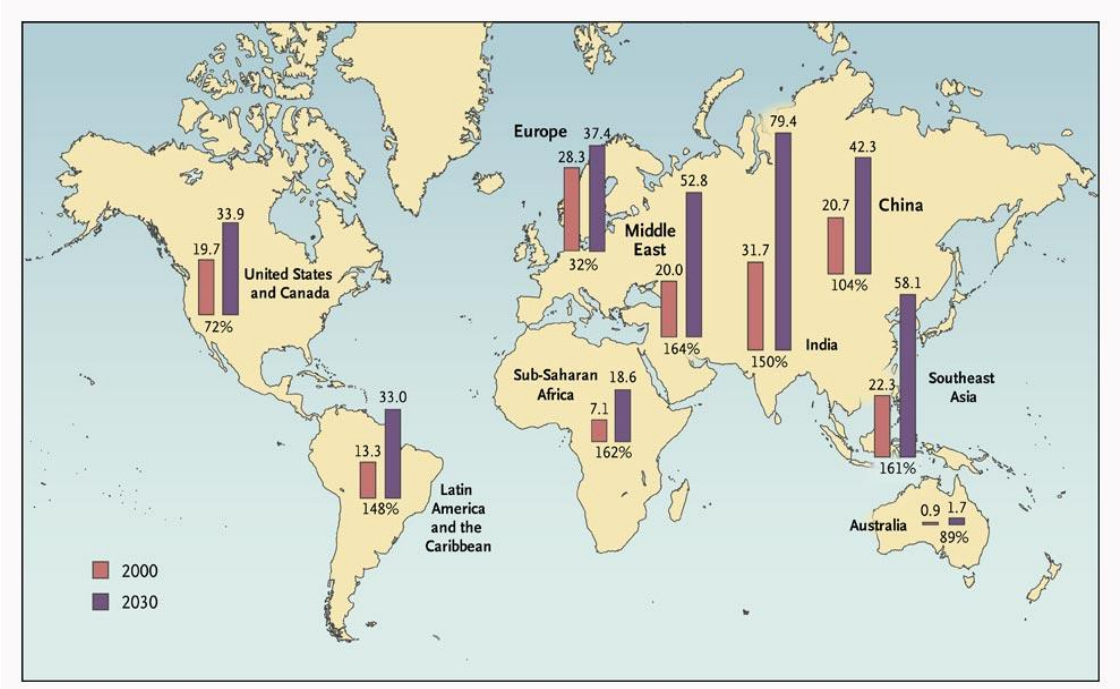

Figure 1.1: Worldwide prevalence of Diabetes Mellitus in 2000 and projections for 2030, with projected percent changes (Hossain, Kawar and Nahas, 2007).

The prevalence of DM is higher in male than in female, but there are more women with DM than men. The combined effect of a greater number of elderly women than men in most populations and the increasing prevalence of diabetes with age is the most likely explanation for this observation. The urban population in developing countries is projected to double between 2000 and 2030. The most important demographic change to DM prevalence across the world appears to be the increase in the proportion of people aged ≥65 years (Wild, Roglic, Green, Sicree and King, 2004).

In Malaysia, prevalence of the DM was 0.65% in the year 1960, 2.1% in 1982, and 4% in 1984 in a limited population based study (Embong, 1990). After that, the first nationwide National Health and Morbidity Survey (NHMS I) was conducted in 1986 and the prevalence was 6.3%, which had risen to 8.2% in the NHMS II in 1996 (Mafauzy, 2006). According to the NHMS III, conducted in 2006, overall prevalence of the DM (known and newly diagnosed) was 11.6% in those above 18 years and 14.9% in those above 30 years. There was an increasing trend in prevalence with age; from 2% in the 18–19 year olds to an alarming prevalence ranging between 20.8–26.2% among the 50–64 year olds. The prevalence was significantly higher among urban compared to rural areas (12.1% vs 10.5%). No gender difference in the prevalence was observed. Based on the ethnicity, the Indians had the highest prevalence (19.9%), followed by the Malays (11.9%) and the Chinese (11.4%). The national prevalence of known DM, newly diagnosed DM and impaired fasting glucose (IFG) amongst Malaysians above 18 years was 7.0%, 4.5% and 4.2% respectively. The overall prevalence of DM among adults above 30 years increased by 80% over a decade (8.3% in NHMS II vs 14.9% in NHMS III) representing an average 8% rise per year. The prevalence of known DM for the same group has increased by 66%

from 5.7% (NHMS II) to 9.5% (NHMS III). The prevalence of newly diagnosed DM increased from 2.5% (NHMS II) to 5% (NHMS III), an absolute rise of 12% per year (Zanariah, Chandran, wan Mohamad, wan Nazaimoon, Letchuman, Jamaiyah, Fatanah, Nurain, Helen Tee and Mohd Rodi, 2008). However, brief report of the last NHMS IV (conducted in 2011) has announced recently. The current prevalence of T2DM in Malaysia is 20.8% (Lee and Zanariah, 2012).

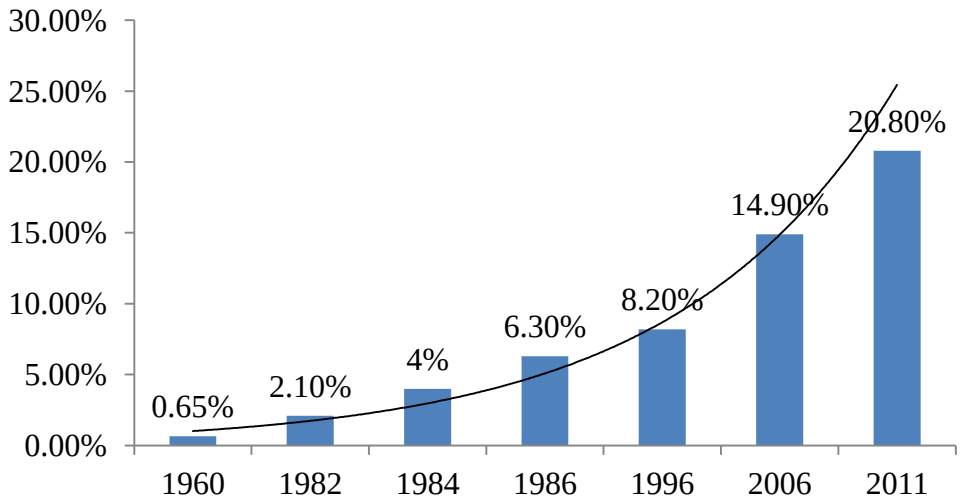

Figure 1.2: Prevalence of Diabetes Mellitus in Malaysia from 1960 to 2011 (Embong, 1990; Mafauzy, 2006; Zanariah et al., 2008; Lee and Zanariah, 2012).

1.1.3 Pathogenesis

Several pathogenic processes are involved in the development of T2DM, which ranges from autoimmune destruction of the β-cells of the islets of Langerhans of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action ("Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus", 2002). Insulin resistance is the commonest pathologic state of T2DM in which target cells fail to respond to the physiologic effects of insulin in peripheral organs and leading to abnormalities in glucose, lipid and protein

metabolism. When the target tissues do not respond to even high levels of insulin, glucose builds up in the blood resulting in high blood glucose. In response to elevated blood glucose concentration, pancreatic β-cells increase further insulin secretion though it is already at high levels to maintain homeostasis in glucose levels. Finally, β-cells become unresponsive to glucose due to pancreatic β-cells dysfunction and eventually T2DM develops (Ahmed, Muniandy and Ismail, 2010).

1.1.4 Diagnosis

According to the American Diabetes Association (ADA), current criteria for the diagnosis of T2DM are as follows:

i. Glycohemoglobin (HbA_1C) ≥6.5%: The test should be performed in a laboratory using a method that is National Glycohemoglobin Standardization Program (NGSP) certified and standardized to the Diabetes Control and Complications Trial (DCCT) assay.
ii. Fasting Blood Glucose ≥126 mg/dl (7.0 mmol/L): Fasting is defined as no caloric intake for at least 8 hours.
iii. 2–hours after plasma glucose ≥200 mg/dl (11.1 mmol/L) during an oral glucose tolerance test (OGTT). The test should be performed as described by the World Health Organization (WHO) using a glucose load containing the equivalent of 75 gm of anhydrous glucose dissolved in water.
iv. In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis: a random plasma glucose ≥200 mg/dl (11.1 mmol/L) (Care, 2010).

The classical clinical features of T2DM are polyuria (frequent urination), polydipsia (unusual thirst), weight loss (unexplained), sometimes with polyphagia (extreme hunger), and blurred vision; sometimes it may present with some unusual

symptoms like nausea and vomiting, extreme weakness and tiredness, irritability, change of mood etc. (Modak et al., 2007). T2DM is diagnosed and confirmed when fasting plasma glucose is equal or higher than 7.0 mmol/L (126 mg/dl) and/or the 2–hours value in an oral glucose tolerance test (OGTT) is equal or higher than 11.1 mmol/L (200 mg/dl). In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose or 2–hours after glucose loading value of 11.1 mmol/l or more (≥200 mg/dl) is enough to confirm the diagnosis (Ministry of Health, 2009).

1.1.5 Diabetes and Dyslipidemia

The characteristic pattern of lipoproteins abnormalities in T2DM includes an increase in triglycerides (TG) and a decrease in High Density Lipoprotein (HDL) without a significant change in Low Density Lipoprotein (LDL) concentration. This triad of lipid abnormalities has been termed "Diabetic Dyslipidemia" (Lalonde, O'Connor, Joseph and Grover, 2004). The mean concentrations of LDL cholesterol in those with T2DM are not significantly different from individuals who do not have diabetes. However, qualitative changes in LDL cholesterol may be present. In particular, patients with T2DM tend to have a higher proportion of smaller and denser LDL particles, which are more susceptible to oxidation and may thereby increase the risk of cardiovascular events (Haffner, 2004).

Another common alteration of lipoprotein in T2DM is an elevation of Very Low Density Lipoprotein (VLDL), as reflected by either increased total TG or VLDL–TG concentration; several studies have suggested that diabetes may have a large TG–rich VLDL. This alteration in VLDL composition may be due to changes in the distribution of the smaller apolipoproteins (apo); apolipoprotein C (apoC) that

control the activity of Lipoprotein Lipase (LPL) and apolipoprotein E (apoE) that influences the affinity for binding to receptor of different cells (Howard, 1987). The exact mechanism is not clear, but there are some explanations:

i. Overproduction of triglyceride increase VLDL as a result of the increased flow of substrates, particularly glucose and free fatty acids to the liver in T2DM patients (Howard, 1987).

ii. Defective and reduced conversion of TG–rich VLDL into LDL and HDL due to deficiency of LPL (an insulin dependent enzyme) activity in the state of insulin resistance of T2DM (Howard, 1987).

iii. Reduced removal from the circulation due to defective receptor binding by apoE, especially in apoE2 that is controlled by ε2 allele (Walden and Hegele, 1994).

Changes in the HDL concentrations in T2DM are due to reduced activity of LPL as well as over activity of hepatic lipase. Reduced LPL activity causes reduced production of HDL and over activity of hepatic lipase causes increased removal of HDL from the circulation. Along with plasma HDL concentration, qualitative changes also occur. This altered composition may in turn alter its 'anti-atherogenic' properties (Howard, 1987).

1.1.6 Molecular Research on Diabetes Mellitus

The rapidly increasing prevalence of T2DM is thought to be due to environmental factors, such as increased availability of food and decreased opportunity and motivation for physical activity, acting on genetically susceptible individuals. The heritability if T2DM is one of the best established among common diseases and, consequently, genetic risk factors for T2DM have been the subject of intense research

(Sladek, Rocheleau, Rung, Dina, Shen, Serre, Boutin, Vincent, Belisle, Hadijadj, Balkau, Heude, Charpentier, Hudson, Montpetit, Pshezhetsky, Prentki, Posner, Balding, Meyre, Polychronakos and Froguel, 2007). Several human monogenic forms of diabetes have been identified including: maturity onset diabetes of the young (MODY, caused by mutation of glucokinase gene), the diabetes–deafness and optic atrophy syndrome (defects in mitocondrial gene), latent adult onset autoimmune diabetes (LADA) (Ahmed et al., 2010), and diabetes secondary to rare genetic disorders; but the ethnic and geographic difference of this disease occurrence indicate that the T2DM is an extremely heterogenous disorder (Gerich, 1998; Li, Isomaa, Taskinen, Groop and Tuomi, 2000).

Although the pathogenesis of T2DM is controversial, it is generally agreed that: (1) the disease has strong genetic and environmental components, (2) its inheritance is polygenic meaning that the simultaneous presence of several abnormal genes or polymorphisms is necessary for development of disease, (3) impairment of insulin sensitivity and insulin secretion, each of which are under genetic control, are both important elements in its pathogenesis, (4) most patients are obese, and (5) obesity, especially abdominal obesity, causes insulin resistance and is also under genetic control (Gerich, 1998).

Like other common complex diseases, hunt for the T2DM–susceptible genes started a few decades ago. Over this period, study design evolved as a result of progress in technology, in the understanding of the patterns of human genome sequence variation and in the availability of appropriate-ascertained samples. Hundreds of candidate gene studies and more than 30 genome wide linkage scans for T2DM have been published (McCarthy and Zeggini, 2009) but none of them were successfully identified as T2DM susceptible loci. Eighteen single nucleotide