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New susceptibility loci associated with Type 2 Diabetes

By

Nadine A. Mastouri

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Student Name: Nadine Mastouri

I.D. #: 200802838

Thesis Title : **New Susceptibility Loci Associated with Type 2 Diabetes**

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School : **School of Arts and Sciences**

Approved by:

Thesis Advisor: Dr. Pierre Zalloua

Member : Dr. Sima Tokajian

Member : Dr. Ralph Abi Habi

Member :

Date

(This document will constitute the first page of the Thesis)

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Signature:

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New susceptibility loci associated with Type 2 Diabetes

Nadine A. Mastouri

Abstract

Type 2 Diabetes (T2D) is a complex multifactorial disease, characterized by insulin resistance and a progressive pancreatic β cell failure, leading to an increase in fasting plasma glucose level. Up to date and despite the tremendous evolution in genetic research, less than 10% of T2D underlying genetic basis has been identified. Hence the whole picture of T2D inheritance is not completely exposed yet. So far, the mutations recognized, either through the candidate gene approach or recently through the Genome wide association studies, are positioned either in genes regulating the insulin resistance pathway or secretion, or in segments of genes of unknown function.

From the most recent mutations identified in 2010, we selected three genes, the Hepatocyte Nuclear factor 1-A (HNF1A) (rs7957197), the High mobility group 2 (HMGA2) (rs1531343) and the Kruppel-like Factors 14 (KLF14) (rs972283) to be the material of our study. Our aim was to identify the polymorphisms related to these genes in the Lebanese diabetic population. 1205 patients were selected (565 diabetic, 640 control subjects) and were genotyped for polymorphisms in HNF1A gene, HMGA2 gene and KLF14 gene. Statistical analysis using chi-square test was utilized to compare the genotype and allele frequencies between the two groups of the study. Our analysis revealed an association between the TT genotype of HNF1A-rs7957197 and T2D in the Lebanese diabetic population ($P = 0.028$) but no significant correlations were detected for KLF14-rs972283 and the HMGA2-rs1531343 ($P = 0.27$ and 0.58 respectively). Furthermore, when considering the treatment with either insulin or an Oral hypoglycemic agent, significant results were observed in HNF1A-rs7957197, where the risk allele T is more common in patients treated with OHA as compared either to a different type of treatment ($P = 0.04$) or to treatment with insulin injections ($P = 0.03$). Similarly, in KLF14-rs972283 polymorphism the genotype GG was found to be correlated with diabetic patients not treated with insulin injections ($P = 0.02$).

In conclusion, the HNF1A-rs7957197 and KLF14-rs972283 mutations could be involved in attenuating the rate of β cell failure. Further studies with a larger Lebanese sample size must be accomplished to confirm our results.

Keywords: Type 2 Diabetes, New loci, β cell failure, Treatment with insulin.

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GLOSSARY

AF-1: Activation function domain 1

BMI: Body mass index

CI: Confidence interval

FTO: Fat mass– and obesity associated gene

FFA: Free fatty acids

Glp-1: Glucagon like peptide -1

Glp-2: Glucagon like peptide -2

GLUT 2: Glucose transporter 2

GLUT 4: Glucose transporter 4

GWAS: Genome-wide association studies

HDAC: Histone Deacetylases

HDL: High density lipoproteins

HMGA2: High Mobility Group A 2

HNF1 α : Hepatocyte Nuclear Factor 1 α

HOMA- β : Homeostasis model assessment of β cell function

HOMA-IR: Homeostasis model assessment of insulin resistance

IGFBP2: Insulin-like growth factor binding protein 2

IGF2: Insulin growth factor 2

IL-6: Interleukin-6

KLF14: Kruppel-like Factor 14

LIPE: Lipase, Hormone sensitive enzyme

LDL: Low density lipoproteins

MODY: Maturity onset diabetes of the young

OASL: 2'-5'- oligoadenylate synthetase-like

OR: Odds ratio

OHA: Oral hypoglycemic agents

PPARG: Peroxisome proliferate-activated receptor γ

ROS: Reactive Oxygen Species

SCL30A8: Solute carrier family 30 member 8

SGLT2: sodium dependent glucose transporter 2

SNP: Single nucleotide polymorphism

SP1: Specificity protein 1

T2D: Type 2 Diabetes Mellitus
TCFL2: Transcription factor-7-like 2
TGF β : Transforming growth factor β
TGF β RI: Transforming growth factor β receptor I
TGF β RII: Transforming growth factor β receptor II
TNF α : Tumour necrosis factor α
ZnT8: Zinc transporter-8
VLDL: Very low density lipoproteins

CHAPTER ONE

INTRODUCTION

1.1. Prevalence of Type 2 Diabetes

According to the WHO 1999 criteria, Type 2 diabetes (T2D) is a disease characterized by an elevated fasting plasma glucose concentration of 126 mg/dl and more (Giorgino, Laviola & Leonardini, 2005). Worldwide, over 170 million individuals have T2D, and this is expected to increase to 370 million by the year 2025 (Doria, Patti & Khan, 2008; Holt, 2004). In harmony with this pattern, the prevalence of diabetes in Lebanon has been regularly increasing with time. Being 7-8% in adults over 30 years of age in 1969, the frequency increased to 13.1% in 1997 (Salti, Khogali, Alam, Abu haidar & Masri, 1997), to 15.8% in 2005 (Hirbli et al. 2005) and to 18.9% of adults above 40 years old in 2007 (Kristensen, Bak, Wittrup & Lauritzen, 2007).

Family, twins and population studies have confirmed that 30-70% of T2D risk is due to genetic factors. Monozygotic twins have double the concordance rate for T2D as compared to dizygotic twins, and the offspring of one diabetic and two diabetic parents show a risk of 35% and 70% respectively, compared to 10% of the population. These facts constitute a solid proof regarding the relation between T2D and the genetic predisposing factors (Walford & Florez, 2010).

At the same time, environmental factors and life style, mainly obesity and physical inactivity, have an accentuating impact on the disease development (Walford & Florz, 2010; Holt, 2004). The constant increase in diabetes frequency worldwide, can also be attributed to an elevation in the obesity rate: in the united states, 67% of patients with diabetes have a BMI of more than 27kg/m² and 46% more than 30kg/m² (Holt, 2004).

Hence, diabetes is a multifactorial heterogeneous disease, caused by an interaction between several genes each of which has, besides the environmental factors, a precise effect on the disease development (Walford & Florez, 2010).

1.2. Pathophysiology of T2D

The disease pathophysiology is caused by a peripheral insulin resistance (failure of peripheral tissues to respond to insulin) and insulin deficiency due to a decrease in β cell functions (Giorgino et al. 2005; Lupi & Del Pato, 2008). The initial event in the disease is characterized by a deficit in insulin action, followed by an impaired insulin secretion (Giorgino et al. 2005).

Figure 1: The interface of environment and genetics with T2D pathogenesis (Giorgino et al. Pathophysiology of type 2 diabetes: Rationale for different oral antidiabetic treatment strategies. *Diabetes research and clinical practice*. 2005; 68S1:S22-S29).

1.2.1. Insulin resistance

Insulin regulates glucose metabolism in the muscles, liver and adipose tissues (Luca & Olefsky 2007). In the skeletal muscles, it induces glucose uptake in the cell through the transfer of glucose transporter 4 (GLUT4) to the plasma membrane. In the liver, insulin prevents the activity of the gluconeogenic enzymes, and in the adipose tissues, it decreases the activity of the lipase Hormone sensitive enzyme (LHPE), causing less hydrolysis of Triglycerides resulting in a decrease in the formation of free fatty acids (FFA) and their release from the adipocytes into the circulation (Luca & Olefsky, 2007).

a. *Genetic factors*

Insulin resistance is related to environmental factors such as obesity, aging, smoking, low physical activity and to underlying genetic causes (Mlinar, Marc, Janez & Pfeifer, 2007). The Genome wide associations studies (GWAS) have identified the Peroxisome proliferate-activated receptor γ (PPARG) gene that alters insulin sensitivity. The latter gene is located on the short arm of

chromosome 3. It regulates directly the genes involved in lipid metabolism, uptake and storage, and glucose metabolism (Jeninga, Gurnell & Kalkhoven, 2009). PPARY, when activated, induces the secretion of adipopectin hormone, through the activation of adipopectin gene transcription and that increases insulin sensitivity (Jeninga et al. 2009). The mutation from Proline to Alanine at position 12 (Pro12Ala) is located in the activation function domain 1 (AF-1), within the N-terminal segment of the gene, and it induces insulin resistance through a decrease in the target gene activation (Jeninga et al. 2009). Depending on the ethnic group, the frequency of this most common replicated single nucleotide polymorphism (SNP) in diabetes varies between 2-25% (Jeninga et al. 2009; Ridderstråle & Groop, 2009).

b. Inflammation and obesity

Obesity, mainly visceral adiposity, a main element in insulin resistance process, is associated with an inflammatory response. An increase in adipose tissues, caused by hypertrophy and hyperplasia of adipocytes, results in a decrease in the oxygen supply vs demand, causing cell hypoxia of adipocytes thus launching an inflammatory pathway. This process involves recruitment of macrophages and the secretion of cytokines such as interleukin-6 (IL-6), IL-8 and Tumour necrosis factor α (TNF α) (Luca & Olefsky, 2007; Giorgino et al. 2005); the latter being the main pro inflammatory cytokine involved in insulin resistance related to obesity (Mlinar et al. 2007).

The large amounts of TNF α released from the adipose tissues, provoke the secretion of FFA in the circulation, the excess of which will affect the different organs that constitute the target territory where insulin performs its functions (Mlinar et al. 2007).

In the muscles, elevated levels of FFA, foster beta oxidation that in turns, results in diminishing glucose oxidation and uptake, and inhibit glycogen synthesis (Mlinar et al. 2007). In the adipose tissues, they reduce the lipoprotein lipase activity, preventing the disposing of FFA from the blood. In the liver, gluconeogenesis takes place simultaneously with the synthesis of triglycerides and very low density lipoproteins (VLDL) from FFA (Mlinar et al. 2007).

Figure 2: The effect of obesity on different organs and the development of insulin resistance (Luca & Olefsky, Inflammation and insulin resistance. *Federation of European Biochemical Societies*. 2007; 582: 97-105).

1.2.2. Pancreatic β cell failure

β cell failure that leads to a progressive decrease in insulin secretion is the result of environmental and genetic factors. The main underlying reason is the apoptosis of β cells that causes a reduction in β cell mass. This is caused by glucotoxicity, where the chronic elevation of glucose level increases the apoptosis rate of β cells through the generation of reactive oxygen species (ROS) (Lupi & Prato, 2008). Also by lipotoxicity due to the high levels of FFA in the blood and their generation of Nitric oxide and by an increase in amyloid deposition in the pancreatic β cells, considered to be toxic for these cells (Lupi & Prato, 2008).

a. *Genetic elements*

Several genes such as Transcription factor-7-like 2, TCFL2, and solute carrier family 30 member 8, SLC30A8, have been identified to have an effect on β cell functions.

Transcription factor-7-like 2, TCFL2 gene, is one of the first susceptibility genes for T2D to be replicated by GWAS (Doria et al. 2008). It is located on

chromosome 10q25 and is involved in WNT signaling pathways as the β catenin nuclear receptor. This pathway is essential for embryogenesis and pancreatic Islet cell development (Ridderstråle & Groop, 2009 ; Doria et al. 2008). The binding of TCFL2 to the β catenin nuclear receptor causes the transcription of several genes such as intestinal proglucagon, glucagon like peptide-1 (GLP-1) and glucagon like peptide -2 (GLP-2) (Ridderstråle & Groop, 2009) responsible for the regulation of glucose homeostasis. The rs7903146 in TCFL2 with T as the risk allele is linked to diabetes (Owen et al, 2007). The mutation in TCFL2 gene inhibit the positive stimulatory effect of both GLP-1 and 2 on insulin, causing a decrease in insulin secretion from pancreatic β cells (Ridderstråle & Groop, 2009). In different ethnic groups, carriers of the risk allele have a 30%-50% increased risk to develop T2D (Hattersley, 2007).

SCL30A8, solute carrier family 30 member 8 gene, located on chromosome 8q24.11, is a member of the Zinc transporter family (Xu et al. 2010). The gene is formed of eight exons and codes for a 369 amino acid protein, known as the Zinc transporter-8 (ZnT8) (Xu et al. 2010). This transporter has a major role in Zinc transport from the cytoplasm into the insulin vesicles of the nuclei of pancreatic islets cells (Xu et al. 2010). The zinc is essential for insulin maturation from proinsulin to insulin, storage and secretion (Xu et al. 2010). A missense mutation, rs1326634 C/T, altering arginine to tryptophan at position 325 (R325W), is implicated in T2D, by affecting zinc transfer (Xu et al. 2010; Doria et al. 2008).

Figure 3: A display of all the factors involved in T2D pathogenesis (Jin & Patti, Genetic determinants and molecular pathways in the pathogenesis of type 2 diabetes. *Clinical Science*. 2009. **116**, 99-111).

1.3. Approaches in gene discovery

From spring 2010, more than 40 different loci have been linked to T2D (Walford & Florez, 2010). Initial studies were made possible through linkage analysis that identified monogenic forms of diabetes such as Maturity onset diabetes of the young (MODY), neonatal diabetes, both representing 2-5% of diabetic cases (Majithia & Florez, 2009). That was then followed by the candidate gene scheme used to detect mutations in genes known to have a role in the pathophysiology of the disease (Walford & Florez, 2010). Recently, these two methods were complemented by GWAS. GWAS was made possible after the sequencing of the human genome, and it allowed the detection of SNP in genes with no prior biological knowledge of these specific genes (Grarup, Sparso & Hansen, 2010).

1.4. Functions of the new genes

All the genes identified and proved to be associated with diabetes represent less than 10% of T2D genetics. Three new SNP have been identified recently and are considered to be positioned near candidate genes involved in this disease pathogenesis (Voight et al., 2010). These SNP, rs7957197, rs1531343, rs972283, not previously related to T2D, are located near the genes Hepatocyte Nuclear Factor 1 A (HNF1A), High Mobility Group A2 (HMGA2) and Krupel-like factor 14 (KLF14) respectively, and represent the core of this thesis. Hence a description of the nearby genes looks to be an indispensable step in discovering new pathways for T2D pathogenesis.

1.4.1. Hepatocyte Nuclear Factor 1 A (HNF1A)

HNF1-A, a transcription factor responsible for the genes regulation in the liver, kidneys and pancreas, is located on chromosome 12q24.2 and is formed of 10 exons (Ryffel, 2001). Its structure is composed of three functional domains: a dimerization domain at the N-terminal, a DNA binding domain from amino acid 100 till amino acid 281 and a transactivation domain at the C-terminal (Chantelot et al. 2008). HNF1-A binds a conserved sequence, GTTAATTNATTANC constituted of 13 bases located in the promoter segment of the target genes (Ryffel, 2001). In the liver, it controls genes whose products are involved in lipid, carbohydrate metabolism, seroproteins synthesis and in detoxification such as apolipoproteins, aldolase, albumin, and cytochromes respectively (Cereghini, 1996; Fajans, Bell & Polosky, 2001). In the kidneys, HNF1-A affects the sodium dependent glucose transporter 2 (SGLT2), located in the tubular cells (Ryffel, 2001). Accordingly, a mutation in HNF1- α will cause a decrease in glucose reabsorption at the level of proximal tubules resulting in glucosuria (Ryffel, 2001). In the pancreas, HNF1-A regulates the genes coding for insulin, glucose transporter 2 (GLUT 2), pyruvate kinase and more (Pontoglio, 2000), all crucial for the β cells secretory and metabolic functions (Ryffel, 2001).

Moreover, a mutation in this gene is associated with the Maturity onset diabetes of the young type 3 (MODY 3), characterized by a deficit in insulin secretion and a decrease in renal glucose reabsorption (Fajans et al. 2001). More than 200 mutations, classified either as missense or insertion/deletion mutations, have been identified in HNF1- α and linked to MODY 3 (Chantelot et al. 2008).

But recently, it has been proven that a mutation in HNF1- α gene is related to T2D. It is attributed to a very common polymorphism, the substitution of isoleucine to leucine (I27L) located in exon 1, in the dimerization domain of the gene. This mutation induces a small conformational change and affects the DNA binding capacity of the gene. Due to this substitution, the transcriptional activity of the GLUT 2 gene is reduced (Chen et al. 2010). Thus, less glucose will be transported into the β cells, and ultimately less insulin will be secreted (Fajans et al. 2001). So I27L causes a dysfunction in β cells, influencing insulin secretion and thus increasing the risk of T2D (Chen et al. 2010). Therefore, unrelated mutations on HNF1- α gene may either cause a monogenic form of diabetes or induce the most common form of diabetes, T2D (Chiu, Chuang, Chu, Yoon & Wang, 2003).

Furthermore, mutations in HNF1 α gene are related to the C- reactive protein (CRP) concentration in the plasma (Reiner et al. 2008). This inflammatory protein is mainly produced by liver cells and its transcription is controlled by IL-6, IL1- β and NF- κ B (Reiner et al. 2008). HNF1-A binds to the promoter of the CRP gene at the same binding site of NF- κ B gene (Reiner et al. 2008). So, HNF1-A can regulate CRP synthesis by the liver (Reiner et al. 2008). One common SNP is the Rs1169310 (G/A), located in the 3'UTR region of HNF1 α and has the strongest association with CRP levels in the blood (Reiner et al. 2008).

The SNP rs7957197, considered a new polymorphism associated with T2D is located on chromosome 12q24.3, in the intron region of the gene 2'-5'- oligoadenylate synthetase-like (OASL) and has T allele as its risk allele (NCBI).

1.4.2. High Mobility Group A2 (HMGA2)

HMGA2, a DNA binding protein, is a member of the High mobility group family A (HMGA) (Fedele & Fusco, 2010). The gene is located on chromosome 12q14, and spans a region on the DNA of at least 160 Kb (Ashar, Tkachenko, Shah & Chada, 2003). It is a nuclear protein, with a mass of less than 30 KDa and implicated in chromatin structural changes and in gene transcription regulation (Bianchi & Agrsti, 2010; Fedele & Fusco, 2010). This gene is formed of three DNA binding domains at the N terminal, known as AT-hooks able to bind to target DNA segment rich in AT

sequences, and of an acidic C terminal essential for protein-protein interactions (Ashar et al. 2003).

In 2006, Hock, Furusawa, Ueda and Michael proved that mice over expressing a truncated form of *hmga2* lacking the C tail, are obese and giant (Hock et al. 2006). Thus HMGA2 has a role in obesity and in height regulation. Anand and Chada proved that in *hmga2*^{-/-} null mice, no difference was detected in weight gain when mice were fed with either normal or high fat diet. Accordingly it is the deletion in *hmga2* allele that prevented the mice from gaining weight (Anand & Chada , 2000).

Additionally, HMGA2 regulates insulin-like growth factor binding protein 2 (IGFBP2) which encodes a protein that binds to the 5'UTR region of the insulin growth factor 2 (IGF2), essential for insulin signaling (Rodriguez et al. 2010). IGFBP2 mutation, rs4402960, influence β cell functions and causes a decrease in insulin secretion (Rodriguez et al. 2010). The mechanism through which IGFBP2 affects these β cells is unknown. It may be that IGFBP2 causes the sequestration of IGF2 mRNA in β cells, ending up in a decrease in β cells volume and in insulin secretion (Cleynen et al. 2010).

Furthermore, the rs4409260 mutation identified in IGFBP2, is positioned within a long haplotype block with isofrequent alleles that spans most of intron 1 and intron 2 of the gene (Rodriguez et al. 2010). And knowing that HMGA2 binds to the AT rich region of IGFBP2 present in the first intron of the gene, it is possible that HMGA2 regulates the alleles of rs4409260, inducing a modification in the function of IGFBP2 and eventually in the expression of IGF2 (Rodriguez et al. 2010).

As already mentioned, and in addition to its involvement in T2D pathogenesis, HMGA2 gene regulates height, especially childhood height. The two SNP identified in the gene are rs1042725 and rs7968682 and are located within and 12 Kb downstream of the 3'UTR segment of the gene respectively (Weedon et al. 2007). With no influence on the birth height, rs1042725 (T/C) affect childhood height from the age of 7 till 11 years, increasing limb length by 0.3 cm and sitting height by 0.2 cm with each C allele (Weedon et al. 2007).

Rs1531343, the SNP of interest, is located on chromosome 12q15 and has C allele as its risk allele (NCBI).

1.4.3. Krupel-like Factor 14 (KLF14)

KLF14, located on chromosome 7q32.3, belongs to the KLF family. This group is constituted of 17 members that regulate cellular proliferation, differentiation and apoptosis (Rodriguez & Martignetti, 2009). Each of the KLF has a distinct function, but they all share a conserved structure. At the N-terminal, is located a variable segment that regulates the transcription of genes. At the C-terminal, three cysteine²/histidine² with zinc fingers constitute the DNA binding segment (Rodriguez & Martignetti, 2009). The zinc fingers are separated by five amino acid residues, TGEKP, conserved among all KLF families (Haldar, Ibrahim & Jain, 2007). These transcription regulator proteins, through their C₂H₂ zinc fingers, are able to bind to specific sequences such as CACC or GC-GT elements, located in the promoter region of their target gene (Aguilar et al. 2007).

In 2009, Truty et al, in their work on pancreatic cancer cells, identified the first function of KLF14, as being the repressor of the transforming growth factor β receptor II gene (TGF β RII) (Truty, Lomberk, Zapico & Urrutia, 2009).

This TGF β RII belongs to the transforming growth factor β (TGF β) signaling pathway. This pathway regulates a number of cellular activities, including cell differentiation, proliferation and apoptosis, with a negative influence on cellular growth (Shi, Massague & Thoma, 2003). The TGF β ligands such as TGF β cytokines and the Anti-Mullerian hormone, bind to the TGFBRII inducing the phosphorylation of the serine residues of the transforming growth factor β receptor I (TGF β RI) and transmitting the signal through the phosphorylation of intracellular SMAD proteins. The latter are transcription regulators elements. And once these proteins are phosphorylated, they are transferred to the nucleus where they regulate the transcription of the gene of interest by binding to the GTCT sequence located in the promoter region of the gene (Shi et al. 2003).

During the period of development, the TGF β IR and TGF β IIR are located in the pancreatic epithelium, and later in the pancreatic islets and ducts (Lin et al. 2009). Also, the regulatory SMAD proteins are present in the pancreas (Lin et al. 2009). Thus, the TGF β signaling pathway is involved in pancreatic diseases (Lin et al. 2009). Such as the SMAD 3 proteins suppresses the insulin gene through their binding to the gene promoter, and thus inhibition of the smad3 signal will cause an increase in insulin

secretion (Lin et al. 2009). As well, an over activation of the TGF β pathway will repress the genes needed for insulin synthesis and glucose metabolism (Lin et al. 2009).

Truty et al. (2009) proved that KLF14 limits the activity of TGF β RII through a negative feedback loop. The binding of the TGF β ligands, such as TGF β 1 to the TGF β RI, activates the TGF β pathway and causes the expression of KLF14 (Truty et al. 2009). Once up-regulated, KLF14 binds to the GC rich sites in promoter region of TGF β RII and competes with SP1. And through its hydrophobic sequence in N-terminal segment, KLF14 binds to Sin3a. The latter is a co-repressor molecule that suppresses the target gene through its interaction with transcription factors such as the KLF and Histone Deacetylases (HDAC) (Truty et al. 2009). All these events will cause a chromatin remodeling of the TGF β RII gene, converting the active to an inactive form of the gene (Truty et al. 2009).

To mention, that SP1 belongs to the SP/KLF family. This group is composed of specificity protein 1 to 4 (SP1-4) sharing the same structure as the other KLF members (Truty et al. 2009). They are involved in regulating signal transduction pathway and considered recently as non-SMAD regulator of TGF β pathway (Truty et al. 2009).

As for the SNP of the study, rs972283 of KLF14 is located on chromosome 7q32 and has C as its risk allele (NCBI).

Figure 4: The mechanism for the Downregulation of TGF β RII gene by the KLF14. (Truty et al. Silencing of the Transforming Growth Factor- β (TGF β) Receptor II by Kruppel-like Factor 14 Underscores the Importance of a Negative Feedback Mechanism in TGF β Signaling. *The Journal of Biological Chemistry*. 2009. **284**, 6291-6300).

CHAPTER 2

MATERIALS AND METHODS

2.1. Profile of the study group

640 non diabetic above 60 years of age and 565 Diabetic, all unrelated Lebanese, previously included in the cardiac study of the Genomic laboratory, represented the population of my study. Controls were defined as having no diagnosis of diabetes, and cases are individuals with a reported diagnosis of T2D. All participants answered a questionnaire a propos of the age, diabetic status, anti-diabetic medication and relevant clinical information such as BMI value, cholesterol level and family history. All subjects, following clinical indication, had a cardiac catheterization performed in Rafic Hariri University Hospital or the American University of Beirut Medical center.

The DNA was extracted from 10 ml of blood previously drawn from each subject and stored at 4°C.

2.2. SNP selection

The SNP rs972283, rs1531343, rs7957197, represent the target of the current study; their specifications according to the TaqMan® SNP Genotyping Assays by Applied biosystems are illustrated in the following table:

Assay ID	NCBI SNP Reference	Nearby gene	Chromosome	Mutation
C__7614445_10	rs972283	KLF14	7	A/G
C__1692218_10	rs1531343	HMGA2	12	G/C
C__29094181_10	rs7957197	HNF1-A	12	A/T

Table 1: Specifications of the SNP of interest

2.3. SNP Genotyping

The technique used in this experiment is the Applied biosystem 7900 HT fast real time PCR system.

40× SNP aliquots were diluted to a 20×working stock solution with a 1×TE buffer of 10 mM Tris-HCl, 1 mM EDTA, and a pH of 8.0.

The stock DNA was diluted with DNase-free water and 10 ng of the diluted DNA was used for each of the TaqMan SNP genotyping assays. The latter reaction requires a DNA concentration ranging from 1 to 20 ng.

The 20 μ L PCR amplification reaction is formed as well of 9.2 μ L of 1 $\times$ TaqMan universal PCR Master Mix, 0.8 μ L of 20 $\times$ SNP genotyping assays that included the forward and reverse primers to amplify the sequence of interest, and two TaqMan minor groove probes to differentiate between the risk and the non risk alleles (TaqMan® SNP Genotyping Assays, Protocol, Applied Biosystems).

The TaqMan probe is a fluorogenic probe, complementary to the target sequence and has a reporter dye at the 5' end (VIC dye linked to allele 1 of the probe, FAM dye linked to allele 2 of the probe) and a quencher at the 3' end. The proximity between the reporter and the quencher prevents the emission of fluorescence from the reporter (TaqMan SNP Genotyping Assays Protocol. Applied Biosystems).

Figure 5: TaqMan probe. Presence of a reporter at the 5' end and of a Quencher at the 3' end (Real Time PCR: The TaqMan Method. Pierce, 2003)

2.4. Steps of the Real time PCR reaction

During PCR reaction, the TaqMan probe and the primers bind to the target DNA sequence. The Taq polymerase adds nucleotide to extend the primers and replicates the DNA strand to which the probe is attached. The 5' exonuclease activity of the enzyme cleaves the probe, separating the Quencher and the Reporter, resulting in the release of fluorescence by the reporter dye (TaqMan SNP Genotyping Assays Protocol. *Applied Biosystems*).

Figure 6: Steps of the Real Time PCR reaction in SNP Genotyping (TaqMan SNP Genotyping Assays Protocol. *Applied Biosystems*. 2006)

The conditions for the PCR reaction are illustrated in **Table 2** (TaqMan SNP Genotyping Assays Protocol . *Applied Biosystems*).

Table 2: Settings for the PCR reaction

AmpliTaq Gold enzyme activation	95°C for 10 min
Denaturation	45 cycles of 95°C for 15 sec
Annealing and extension	45 cycles of 60°C for 1 min

Subsequent to PCR amplification, an endpoint plate read using the 7900HT fast real time PCR system measures the fluorescence in each well. The Sequence Detection System Software plot the values of the measured fluorescence, and determines the alleles identified in each well (TaqMan SNP Genotyping Assays Protocol. *Applied Biosystems*).The results were reconfirmed manually as well.

2.5. Statistical analysis

Chi-square test was used to compare discrete variables, allele and genotype frequencies between cases and controls. The Student's t-test was used to compare continuous variables expressed as means $\pm$ standard deviation. Odds ratio (OR) with 95% confidence interval (CI) were calculated to evaluate the strength of association between the calculated alleles or genotypes frequencies and the clinical data. A two tailed p value of less than 0.05 was considered statistically significant and an Odds ratio of more than 1 was taken as a positive risk.

CHAPTER 3

RESULTS

3.1. Features of studied population

The diabetic status was the main criterion used to classify the population as cases or controls. A comparison was made, presented in **Table 3**, for variables considered as risk factors for T2D between the diabetic and the non diabetic population. The mean age of the study group was lower ($P = 0.0001$) than the control group due to the fact that all subjects of the latter group were aged more than 60 years. The calculated percentages of females and of subjects with a positive family history were higher in the cases as compared to the controls with p values of 0.04 and < 0.0001 respectively.

Glucose level and the body mass index (BMI) of the diabetic group were significantly higher than those of the control group ($P = 0.0001$ in both conditions). No significant differences were detected in the total cholesterol, High density lipoproteins (HDL), Low density lipoproteins (LDL) and Triglycerides of the 2 groups, with p values of 0.33, 0.46, 0.21 and 0.09 respectively. However, the percentage of subjects diagnosed as lipidemic was significantly higher in the cases than in the controls ($P < 0.0003$).

Table 3: Characteristics of the studied population (N=1208).

	Diabetic N = 565	Non Diabetic N = 643	P -values
Age (years)	62 $\pm$ 9.78	70 $\pm$ 6.82	0.0001
Female (%)	40	34	0.04
Family history of diabetes (%)	68	33.20	< 0.0001
BMI (Kg/m ²)	30.22 $\pm$ 5.77	28.58 $\pm$ 4.84	0.0001
Glucose (mg/ dl)	177 $\pm$ 100	112.22 $\pm$ 30.44	0.0001
Lipidemic (%)	57	42	< 0.0003
Total cholesterol (mg/ dl)	202 $\pm$ 131	195.39 $\pm$ 104.88	0.33
HDL (mg/ dl)	61 $\pm$ 143	55.60 $\pm$ 111.29	0.46
LDL (mg/ dl)	150 $\pm$ 176	138.35 $\pm$ 149.06	0.21
Triglycerides (mg/ dl)	409 $\pm$ 1446	284.33 $\pm$ 1142.06	0.09

All means compared by student-t-test

Percentages compared by chi-square test

3.2. The Genotype and Allele frequencies of Diabetic Vs Non Diabetic subjects

The three SNP mentioned above were genotyped and compared between both groups of diabetic and controls. The results did not reveal any significant difference for the genotype and allele frequencies for both of KLF14-rs972283 and HMGA2-rs1531343 with a p value of more than 0.05 for both SNP (P = 0.27 and 0.58 respectively) (**Table 4**). Whereas for HNF1A-rs7957197, the diabetic population showed a higher frequency of TT genotype (P = 0.02) with OR: 2.92 (1.23-6.66)

Table 4: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 in diabetic and in control subjects.

	Non Diabetic	Diabetic	P value
<i>rs1531343</i>			
GG	380	350	0.27
CC	33	25	
GC	210	159	
G	970	859	0.12
C	276	209	
<i>rs7957197</i>			
AA	335	315	0.02
TT	8	22	
TA	147	144	
A	817	774	0.09
T	163	188	
<i>rs972283</i>			
AA	112	128	0.58
GG	45	41	
GA	123	144	
A	347	400	0.49
G	213	226	

3.3. The Genotype and Allele frequencies of Female Diabetic Vs Male Diabetic subjects

The diabetic population was subdivided into males and females. The data analysis of this group showed no significant differences between both genders, corresponding to the three SNP, with a P value > 0.05 in all cases (**Table 5**).

Table 5: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 in Female diabetic and in Male diabetic subjects.

Diabetic			
	Female	Male	P value
<i>rs1531343</i>			
GG	135	215	0.81
CC	10	15	
GC	66	93	
G	336	523	0.58
C	86	123	
<i>rs7957197</i>			
AA	124	191	0.5
TT	6	16	
TA	58	86	
A	306	468	0.56
T	70	118	
<i>rs972283</i>			
AA	54	74	0.53
GG	19	22	
GA	54	90	
A	162	238	0.95
G	92	134	

3.4. The Genotype and Allele frequencies of Diabetic with a positive or negative Family history for T2D

A comparative study, of the positive T2D family history and the negative T2D family history subjects that constitute the diabetic group, proved no discrepancy between the genotype and allele frequencies of the three SNP, HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 with P values of 0.39, 0.36 and 0.72 respectively. (**Table 6**)

Table 6: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283, in diabetic with a positive or negative family history for T2D.

Diabetic			
	No Family History	Positive Family History	P value
<i>rs1531343</i>			
GG	105	241	0.39
CC	10	13	
GC	52	107	
G	262	589	0.23
C	72	133	
<i>rs7957197</i>			
AA	106	205	0.36
TT	9	13	
TA	41	102	
A	253	512	0.69
T	59	128	
<i>rs972283</i>			
AA	40	86	0.72
GG	12	28	
GA	39	104	
A	119	276	0.62
G	63	160	

3.5. The Genotype and Allele frequencies of Diabetic with BMI < 30 AND BMI ≥ 30

Considering obesity as a main risk factor for T2D, genotype and allele frequencies were calculated for the three polymorphisms. No positive association was detected between these mutations and BMI values. The P value was more than 0.05 in the three cases (**Table 7**).

Table 7: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 in diabetic with BMI less or more than 30

Diabetic			
	BMI < 30	BMI ≥ 30	P value
<i>rs1531343</i>			
GG	166	159	0.25
CC	15	10	
GC	89	63	
G	421	381	0.1
C	119	83	
<i>rs7957197</i>			
AA	162	133	0.51
TT	13	8	
TA	68	67	
A	392	333	0.81
T	94	83	
<i>rs972283</i>			
AA	63	59	0.94
GG	19	18	
GA	65	66	
A	191	184	0.87
G	103	102	

3.6. The Genotype and Allele frequencies of Diabetic with respect to the treatment with either insulin or with Oral hypoglycemic agents (OHA)

When accounting for the protocols used to manage and control diabetes, and when evaluating A vs. T being the risk allele, rs7957197 revealed significant variation in the allele frequencies between diabetic patients treated with any OHA and patients treated with insulin. Patients of the former group were found to have a higher T allele ($P = 0.03$) than diabetic patients treated with insulin (**Table 8**).

A noteworthy finding ($P = 0.04$) was noticed in allele frequencies of rs7957197, among subjects not under insulin injections therapy. These patients were found to have a higher T allele, in contradistinction with subjects treated with insulin injections (**Table 9**).

With regards to the rs972283 polymorphism, significant differences ($P = 0.02$) in genotype frequencies were identified among patients under insulin and patients following different strategies of treatment (**Table 9**). An association was found between

no need for insulin injections in treatment and the GG genotype of the KLF14-rs972283.

Table 8: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 in diabetic with respect to treatment.

Diabetic			
	OHA	Insulin	P value
<i>rs1531343</i>			
GG	160	31	0.31
CC	8	3	
GC	75	22	
G	395	84	0.13
C	91	28	
<i>rs7957197</i>			
AA	148	25	0.06
TT	8	5	
TA	66	16	
A	362	66	0.03
T	82	26	
<i>rs972283</i>			
AA	53	11	0.86
GG	19	3	
GA	71	16	
A	177	38	0.83
G	109	22	

Table 9: Genotype and allele frequencies for HMGA2-rs1531343, HNF1A-rs7957197 and KLF14-rs972283 in diabetic under insulin therapy or treated differently.

Diabetic			
	Insulin	no insulin	P value
<i>rs1531343</i>			
GG	31	219	0.32
CC	3	17	
GC	22	99	
G	84	537	0.21
C	28	133	
<i>rs7957197</i>			
AA	25	202	0.08
TT	5	12	
TA	16	95	
A	66	499	0.04
T	26	119	
<i>rs972283</i>			
AA	11	82	0.02
GG	3	27	
GA	16	93	
A	38	257	0.96
G	22	147	

CHAPTER 4

DISCUSSION

The continuous identification of new susceptibility genes associated to T2D appears to be an active process and remains a vital challenge; it is considered to be the only route to elucidate the complex pathogenesis underlying the development of the T2D. The latter is a polygenic, complex disease, caused by a combination of a genetic predisposition and environmental factors; positive family history, obesity and physical inactivity are considered essential risk factors for this disease.

The goal of this study is to identify the polymorphisms, rs7957197 in HNF1A, rs1531343 in HMGA2 and rs972283 in KLF14 in the Lebanese diabetic population with an emphasis to discover a correlation of these mutations with the risk factors and treatment of T2D.

Our group of study was divided between controls, selected as subjects with no known history of T2D, and cases chosen as individuals with a confirmed diagnosis of T2D.

The TT genotype of rs7957197 in HNF1A, with T as the risk allele, is associated to T2D in the Lebanese population ($P = 0.028$). But the genotype and allele frequencies of rs1531343 in HMGA2 and rs972283 in KLF14 did not show a significant difference when comparing between nondiabetic and diabetic subjects.

Voight et al. (2010), did establish an association between these three SNP and T2D, in 34,412 cases and 59,925 controls, all of European descent, from Germany, UK, Italy and other countries (Voight et al. 2010). But in their study, they performed a meta-analysis of eight GWA of T2D to identify common polymorphisms of weak impact associated with this disease. In addition to their larger sample size, they did genotype 2,426,886 SNP, thus amplifying the probability to detect any polymorphism linked to T2D. They were able to identify 12 new SNP, out of which only HNF1A, HMGA2, KLF14, were considered to be essential candidate genes that increase the susceptibility to T2D.

Also, the non replication of two of these SNP in the Lebanese diabetic population is caused by several factors. First, allele frequencies vary within the same population

and between different populations (Cardon & Palmer, 2003). As well, gene-environment interactions, genetic and environmental heterogeneity between populations represent the main reasons behind the lack of reproducibility between studies (Cardon & Palmer, 2003). Other potential risk factors responsible for the irreproducibility of results are population stratification and publication of only positive findings (Cardon & Palmer, 2003).

Regarding the studied population, the diabetic group did have a higher occurrence of a positive ancestry for T2D, and higher means for BMI and blood glucose level (**Table 3**). Based on the available records, and on the lack of comprehensive data regarding the lipid profile for all studied subjects, no significant differences were detected regarding the total cholesterol, LDL, HDL and triglycerides, between the cases and controls. However, a significant dissimilarity was detected when comparing between the percentages of diabetic lipidemic and non diabetic lipidemic.

Dyslipidemia in diabetic patients is characterized by high plasma triglycerides, a low HDL and a high LDL levels in the blood (Mooradian, 2009). These features of dyslipidemia are related to the insulin resistance of T2D, with the main cause being the high levels of circulating FFA (Mooradian, 2009). The Framingham Heart Study did reveal that 19% of diabetic males and 17% of diabetic women have elevated concentrations of triglycerides as compared to 9% and 8% of non diabetic men and women respectively. Yet, diabetics from both genders have similar total cholesterol and LDL concentrations as non diabetic men and women (Mooradian, 2009). Furthermore, the frequency of low HDL was much more common in diabetic men and women (21% and 25% respectively) as compared to non diabetic males and females (12% and 10% respectively) (Mooradian, 2009).

The frequency of dyslipidemia is not increased in diabetic patients, but when present, it adversely affects the mortality rate from cardiovascular conditions (Mooradian, 2009). But our results indicated a higher percentage of diagnosed lipidemic in cases than controls.

As for the gender, no significant statistical differences ($P > 0.05$) were detected between diabetic males and females for rs7957197 in HNF1A, rs1531343 in HMG2 and rs972283 in KLF14. It is documented that the incidence of T2D is almost similar between males and females (Franconi et al. 2008). But, diabetic females unlike diabetic

males, have an isolated postprandial hyperglycemia rather than a fasting hyperglycemia (Franconi et al. 2008). So a proper diagnosis of T2D in females based on fasting hyperglycemia can cause an “under-diagnosis” of diabetic females (Franconi et al. 2008).

However, differences between the two genders are detected in the associated cardiovascular conditions. Diabetic females have a 4-6 fold increase in the risk to experience a coronary artery disease compared to a 2-3 fold increase for diabetic male (Legato et al. 2006). Also, diabetic females have a 50% higher chance to suffer from a fatal heart attack (Franconi et al. 2008).

Furthermore, despite the fact that a positive family history of T2D is the foremost risk factor for the disease development, no significant divergence was observed when the population of our work was subdivided according to the presence or absence of a family history of T2D ($P > 0.05$ in the three mutations).

An African study revealed that individuals with a positive family history of T2D have higher levels of blood glucose and a 4 fold increased risk to develop diabetes (Bochud et al. 2004). Furthermore, Bochud et al. (2004) studied the analytical validity, sensitivity and specificity of the component of the family history of T2D, considered to be an essential element used in screening projects that aim to detect the incidence of a particular disease in a family. The sensitivity represented the rate of individuals who reported the presence of disease in truly affected siblings, whereas specificity constituted the proportion of subjects who reported the absence of the disease in really unaffected siblings. Consequently, it was established that the sibling history of diabetes had a low sensitivity (53.4%) and a high specificity (98.2%). Hence, these results reveal that the family history of diabetes is a weak screening tool to forecast diabetes in siblings.

In the population of our work, 93% of diabetic subjects had a sibling or parent with the disease, 5% had diabetic cousins or aunts, and the rest had grandparents with the disease. But such data, despite the high specificity as mentioned earlier, does not reflect the true T2D heritability with regards to the link between the family history and the mutations of rs7957197 in HNF1A, rs1531343 in HMGA2 and rs972283 in KLF14.

Moreover, considering the analysis done based on BMI, the allele and genotype distributions of the three polymorphisms, did not prove any significant association between obesity, a BMI >30, with the higher risk to develop T2D ($P > 0.05$ for all three SNP), despite the fact that deletion of HMGA2 was proven to be associated with obesity in mice (Anand & chada, 2000).

This finding is also supported by the study done by Voight et al. (2010). Their sample volume consisted of 21,000 individuals, but still, they did not detect a new gene that may link obesity to T2D. Accordingly so far, the only gene with its risk allele related with obesity and T2D is the fat mass– and obesity associated gene (FTO) the variations of which accentuate the risk for both of the diseases. One common polymorphism, the A allele of rs9939609, where each copy of this risk allele, increases BMI by 0.4 kg/m², and a 1.31 folds higher odds of obesity and higher chances of T2D, 1.25 higher odds for each allele (Freathy et al. 2008).

In the same context, no information is available to elucidate the impact of the probable differences in BMI values that may be observed between the time when the diagnosis was done and the time of our study.

In addition, the treatment options of T2D target the two main defects in the pathogenesis of the disease. As mentioned earlier, the initial phase of the disease is characterized by an insulin resistance, compensated by an increase in insulin secretion by pancreatic β cells. Later, these cells will become dysfunctional and will limit their secretion of insulin. Both of these conditions lead to hyperglycemia (Bailey, 2000).

The treatment options include insulin injections or different classes of oral hypoglycemic agents (Bailey, 2000). The latter, such as Thiazolidinediones, increase the insulin action through its influence on the insulin resistance that will subsequently cause a decrease in fasting and postprandial glucose. On the other hand, sulfonylureas contribution to diabetes management is materialized by directly stimulating the pancreas to produce and secrete more insulin (Bailey, 2000).

Following an expected decrease in pancreatic β cell functions, these oral medications fail to adequately regulate the plasma glucose level; at this point, insulin injections are introduced as a complementary element to the treatment protocol (Bailey, 2000). These injections will elevate plasma insulin level, and that amplifies the peripheral glucose utilization (Bailey, 2000).

In this study, the treatment option with either insulin injections or an oral hypoglycemic agents was used to sub classify the diabetic subjects. The risk allele T of rs7957197-HNF1A, was proven to be more frequent in diabetic individuals treated with OHA as compared to either insulin treatment ($P = 0.03$) or to a treatment without insulin ($P = 0.02$). Similarly, the genotype GG of KLF14-rs972283 was found to be linked to diabetic patient not treated with insulin ($P = 0.02$). This may suggest that both of these mutations may be protective against a severe β cell failure, since these diabetic patients were still able to control the glucose level in the plasma with oral agents only.

However, our results do not contradict the finding obtained with Voight et al. (2010) for KLF14 only. In the latter study, they evaluated the activity of β cell function and insulin resistance through the measurement of the Homeostasis model assessment of β cell function (HOMA- β) and the Homeostasis model assessment of insulin resistance (HOMA-IR) respectively. They proved that only KLF14 is associated with a higher insulin resistance ($p < 0.05$) and no significant correlations were identified for HNF1-A and HMGA2 .

Also, the fasting insulin levels were measured in order to further determine whether these mutations are linked to pancreatic β cell failure or to insulin resistance. They detected an elevated fasting insulin level, in rs972283-KLF14, ($P = 0.029$), implying that this mutation was without a doubt associated with a defect in insulin action at the level of the tissues and not in insulin secretion (Voight et al. 2010). As for rs7957197-HNF1A and for HMGA-2, the fasting insulin levels were measured but with p values of 0.10 and 0.36 respectively, depriving these results from their significance (Voight et al. 2010).

The main distinction between the studies achieved to determine the impact of these mutations in T2D pathogenesis, is the fact that the treatment options constituted the points of repair criteria of our work. Whereas Voight et al. (2010) choose to directly assay the fasting insulin levels, therefore providing a better perspective to more reliable and significant results.

Finally, the main handicap is the small volume of cases and controls enrolled in our study and the small number in the subgroups formed when analyzing the associations between the mutations and the risk factor for T2D. This may reflect an imprecise approximation of the magnitudes of the observed results. Also, the poor

available information on BMI, lipid profile and medication could have limited the effect assessment of these variables in determining their association with the mutations of interest.

CHAPTER 5

CONCLUSION

Type 2 diabetes, a chronic metabolic condition, is the result of the interaction between several genes and the environment. Significant progress has been accomplished lately to identify new genes related to T2D pathogenesis.

In this study, we were able to replicate the SNP rs7957197 in the HNF1- α region, in the Lebanese diabetic population. This polymorphism did not correlate neither with gender, family history of T2D, nor with obesity. However its risk allele, the T allele, was more common in diabetic subjects treated with OHA. As a result and since these patients do not require treatment with insulin injections, this rs7957197 in the HNF1- α region mutation, may be implicated in diminishing the rate of β cells.

The other two SNP, rs972283 in KLF14 region and rs1531343 in HMGA2 region, were not associated with T2D in our population. However, the study revealed that the GG genotype of rs972283 is linked to the Lebanese diabetic patients not treated with insulin injections, implying its role in protection against a severe pancreatic β cell failure.

Since these three SNP have been recently added to the genes linked with T2D pathogenesis, and because of a probable limitation due to our relative small sample size, further studies with a larger Lebanese population need to be accomplished. And this in order to replicate our findings and to highlight further on the risk factors of T2D such as BMI, family history and to explore the effect of the different oral hypoglycemic agents on gene polymorphism.

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